Molecular Detection of *Mycobacterium bovis* in the Environment

Jamie Stuart Young

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Contents

Declaration

I hereby declare that all the results presented in this thesis were obtained by myself under the supervision of Professor Elizabeth M. H. Wellington, unless stated otherwise. This thesis has not been submitted for a degree in any other institution.

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List of Abbreviations

ACE	Abundance-based coverage estimator
ATCC	American Type Culture Collection
BCG	Bacillus Calmette-Guerin
bp	Base pairs
BSA	Bovine serum albumen
cDNA	Complementary DNA
cfu	Colony forming units
DEFRA	Department for Environment, Food, and Rural Affairs
DNA	Deoxyribonucleic acid
dNTP	Equimolar mix of dATP, dCTP, dGTP and dTTP
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EDTA	Ethylenediaminetetra-acetic acid (disodium salt)
G/C	Guanine/Cytosine content
Jack1	First-order Jackknife richness estmator
JCM	Japan Collection of Microorganisms
Kbp	Kilo-base pairs
KPa	Kilo-Pascals
ML	Maximum likelihood
MMmeans	Michaelis-Menten richness estimator (estimators averaged once from means)
MMruns	Michaelis-Menten richness estimator (estimators averaged over runs)
Mbp	Mega – base pairs
mRNA	Messenger ribonucleic acid
NTB	Non-tuberculous bacteria
NJ	Neighbor Joining
OSI	Ordinance Survey Ireland
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PPD	Purified Protein Derivative

PZA	Pyrazinamide
Q-PCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
SOBS	Species observations
TB	Tuberculosis
TCH	Thiopen-2-carboxylic acid hydrazide
TIFF	Tagged image file format

Summary

An investigation was carried out to determine the presence and persistence of *Mycobacterium bovis* in the environment. Soil samples were taken in April 2000 from a farm in Ireland which had undergone a bovine tuberculosis outbreak some four months prior. Total community DNA was extracted from these samples and PCR carried out targeted to two genes specific for the *Mycobacterium tuberculosis* complex; *mpb64* and *mpb70*. These genes were detected in soil samples taken from entrances to two badger sets and in soil from two sites where the infected cattle grazed. Further analysis of DNA using oligonucleotide primers specific for the 16S rRNA genes of slow-growing mycobacteria was carried out. This revealed the presence of 16S rRNA genes relating to *Mycobacterium bovis*. RT-PCR was also carried out using these primers on total community RNA. Sequences relating to *M. bovis* were found, indicating that the DNA and RNA came from viable, intact cells in the soil, and that *M. bovis* persists in soil for up to four months after contamination of the soil. Sampling was repeated in November 2002 following a second TB outbreak in January 2001. DNA sequences for *mpb64* and *mpb70* were only found in the samples from the badger setts, as were 16S rRNA sequences.

The survival of the vaccine strain *M. bovis* BCG was also determined, using soil microcosms experiments in defined environmental conditions. *M. bovis* BCG was found to survive for longest at temperatures of 37°C and at soil wetting levels of 30 %. Culturable cells could not be detected after 60 days, however DNA and RNA relating to *M. bovis* BCG could be detected up to 18 months after initial inoculation. This suggests the cells persisted in a viable non-culturable state. Experiments to determine the rate of persistence of DNA in soil were carried out. DNA was found to persist for no longer than 10 days in soil.

Diversity studies were carried out on the farm samples and on Warwick soil, to determine the diversity of the mycobacterial population. 16S rRNA analysis was carried out and showed the presence of sequences relating to *M. bovis, M. hiberniae, M. avium, M. fallax, and M. farcinogenes.*

Chapter 1: Introduction

1.1. The *Mycobacterium* genus

Members of the *Mycobacterium* genus (Gr. n. *myces* a fungus; Gr. neut. dim. n. *bakterion* a small rod; M. L. neut. n. *Mycobacterium* a fungus rodlet), have been well studied particularly due to certain species e.g. *Mycobacterium avium* subsp. *avium* and *Mycobacterium bovis* being pathogenic to both humans and farmed animals. The mycobacteria are the only members of the family *Mycobacteriaceae*, of the actinomycetes and are aerobic to microaerophilic, bacteria that usually form slightly curved or straight non-motile rods (0.2-0.6 x 1.0-10 μm) (Wayne and Kubica, 1986). They are acid-alcohol fast at some stages of growth and although they do not stain easily using a Gram stain method, they are considered Gram positive. Mycelium-like growth may occur with fragmentation into rods and coccoid elements. Many species form white or cream-coloured colonies, and certain species form yellow or orange carotenoid pigments. Due to the mycolic acid content of the cell wall, the genus is often grouped with closely related genera, including *Nocardia* and *Corynebacterium*. Classification of species is complex and the members of the *Mycobacterium* genus can be grouped according to several different factors including growth rates, pigmentation, fatty acid content and pathogenicity. The primary grouping is based on pathogenicity and places species in two groups: tuberculosis causing bacteria and non-tuberculous bacteria (NBT) (Eisenstadt and Hall, 1995). The NBT are further characterised into slow and rapid (fast) growers. Fast-growers are classified as species which under optimal nutrient and temperature regimes, produce grossly visible colonies from dilute inocula in solid media, in under 7 days. Slow-growers are classified as those species taking over 7 days to give visible colonies, again under optimal temperature and nutrient regimes (Lévy-Férbault and Portaels, 1992). These divisions equate to slow-growers having a culture doubling time of 0.2 doublings or fewer per hour, and fast-growers, 0.2 to 1.0 doublings per hour (Colston and Cox, 1999). This initial grouping was further sub-divided by Runyon (1955), into three groups containing slow-growers and one containing fast-growers, based on pigment production, these groupings were however extremely artificial. These were: Group I - photochromogens (produced pigment in light), Group II - scotochromogens

(produced pigment irrespective of light availability), Group III - nonphotochromogens (did not produce pigments) and Group IV- fast growers.

The genus has expanded greatly since this classification was first published, with novel species being discovered and officially recognized, including; *Mycobacterium wolinskyi* and *Mycobacterium goodii* (Brown *et al.*, 1999) and *Mycobacterium novocastrense* (Shojaei *et al.*, 1997), With the discovery of new species and the fact that pigment production may be temperature dependent and not all strains of a species share pigment producing abilities, these classification schemes have become less reliable. An alternative scheme is based on the pathogenic potential of a species, although this too is constantly changing as pathogenicity is discovered in more species of mycobacteria (Wolinsky, 1979).

The formation of the International Working Group on Mycobacterial Taxonomy (IWGMT) has led to a coordinated approach to determine the taxonomy of the *Mycobacterium* genus. The first cooperative study was on the scotophotochromogenic slow-growers belonging to Runyon group II. The results from this study led to the generation of a frequency matrix for identifying slow-growing mycobacteria in clinical settings. Current classification of the genus is now based on a number of approached including numerical taxonomy, chemo-taxonomy, and molecular analysis.

Although numerical taxonomy has been somewhat superceded by chemo-taxonomy and molecular taxonomy, it still plays an important role in the classification of mycobacteria. The first numerical taxonomy study was by Bojalil *et al.* (1962), and used similar biochemical tests to those used by Runyon. The results gave a good classification of fast-growers, but slow-growing strains proved exceptionally difficult to characterise. Only a small number of phenotypic properties could be analysed and so the classification of the slow-growers was almost identical to the groupings proposed by Runyon.

Tsukamura (1966) introduced a new classification using an Adansonian classification method. Several phenotypic characters of strains were tested including Gram reaction,

acid-fastness, colony morphology, pigmentation, growth rate, reaction to light and catalase activity. Analysis of the S values obtained from collating the results gave five groupings of slow growing species given in Table 1.1.1.

Table 1.1.1. Adansonian classification of slow-growing mycobacteria (Tsukamura, 1966).

Group	Representative members
1	*Mycobacterium tuberculosis, Mycobacterium bovis*
2	*Mycobacterium kansasii*
3	*M. avium*
4	Non-photochromogens
5	Non-photochromogens

Table 1.1.2. Adansonian classification of fast-growing mycobacteria (Tsukamura, 1966).

Group	Representative members
1	*Mycobacterium marinum*
2	*Mycobacterium thermoresistible*
3	*Mycobacterium phlei*
4	*Mycobacterium aurum* and other rapidly growing scotochromogens
5	*Mycobacterium fortuitum* and other rapid growers
6	*Mycobacterium parafortuitum*
7	*Mycobacteriuum smegmatis*

Group 3 was divided into three sub-groups: 3i – non-photochromogenic soil isolates; 3ii – *M. avium* strains and indistinguishable isolates; and 3iii - strains distinguishable from *M. avium.* Fast growers were arranged into seven groups as listed in Table 1.1.2.

Again although further separation was achieved, the basic Runyon groupings remained. Although this Adansonian method can be used to observe similar characteristics of species, and can be used to group them accordingly, phylogeny of the groupings can only be inferred. It gives no evolutionary data and gives groupings as they appear at the time of analysis and not how evolutionary related they are.

The advent of chemo-taxonomy has led to a much better understanding of the relationships of the *Mycobacterium* genus both inter- an intra-generically. The analysis of the cell wall composition of mycobacteria, led to the differentiation of the genus (and other related genera) from other groups within the actinomycetes. As the composition of amino acids, and wall sugars was determined as well as the structure of the peptidoglycan, the genus was characterised as having a cell wall chemotype IV and was placed alongside other genera with the same wall type i.e. *Corynebacterium, Dietzia, Gordona, Nocardia, Rhodococcus* and *Tsukamurella.* Strains belonging to these genera all contain *meso*-diaminopimelic acid, arabinose and galactose and have an A1γ peptidoglycan. They also contain muramic acid residues that are *N*-glycolated rather than *N*-acetlyated (Cummins and Harris, 1958).

All the wall chemotype IV strains contain mycolic acids in the cell wall structure. These are high molecular weight, long chain, 3-hydroxy fatty acids with an aliphatic side chain at position 2. The analysis of mycolic acid content has been of great use in the classification of mycobacteria. Mycobacterial mycolic acids have between 60 and 90 carbon atoms, in a variety of structures, whereas acids in other genera tend to have simple structures and a carbon content of between 22 and 74 atoms. Analysis of fatty-acid, polar lipid and isoprenoid composition has also led to a much better understanding of the genus in terms of the relationship to other genera of the actinomycetes.

Molecular taxonomy has also been used to further classify the genus, in particular the use of 16S rRNA analysis. This has generally underpinned the groupings formed using numerical and chemo-taxonomic methods.

1.2. Diversity within the *Mycobacterium* genus

There is a great deal of diversity within the genus in terms of habitat and, in the case of pathogenic species, in host range. The majority of species are naturally occurring saprophytic organisms, found in soil, marine water, and freshwater environments (Falkinham, 1999). It is believed that all species within the genus share a common ancestor of a fast-growing saprophytic organism, and that evolutionary pressures led to a divergence of particular species into slow-growing animal pathogens (Pitulle *et al.*, 1992). One theory for this divergence is the species became adapted to survival within amoeba species in soil, with the properties acquired leading to the bacteria being able to survive similarly within animal macrophages and leucocytes (Cirillo *et al.,* 1997). Evidence that slow-growing species diverged from the fast growers comes in the form of DNA:DNA analysis between species, and that slow-growing pathogenic species retain genes or partial genes more typically found in saprophytic organisms (Kusunoki and Ezaki, 1992). Phylogenetic analysis of 16S rRNA sequences also confirms this with the members of the slow growers group branching from the fast growers (Brown *et al.*, 1999).

16S rRNA sequences can give an immediate classification for most species in terms of slow or fast growth. The majority of slow growers contain a long helix sequence at position 451-482, which is entirely missing from fast growing species. It is not known if this sequence is from a deletion or an insertion event. There are species on the intersection of the two groups, without the helix, but classified as slow growers. The number of genes encoding for 16S rRNA can also separate slow and fast growers, with the majority of fast growers having two copies of the gene, and slow growers only having one. This has gone some way into explaining the difference in growth rates, although other factors are involved (including promoter strength) (Spratt *et al.*, 2003). These

differences in growth rates and pathogenicity of species (in human disease) are listed in Table 1.2.1.

Table 1.2.1. Characteristics of several *Mycobacterium* species.
(Adapted from Leclerc *et al.*, 2003)

Species	**Growth rate and helix**	**Human pathogenicity**
M. genavense	Slow growth with short helix	Potential pathogen
M. heidelbergense	Slow growth with short helix	Potential pathogen
M. intermedium	Slow growth with short helix	Potential pathogen
M. interjectum	Slow growth with short helix	Potential pathogen
M. lentiflavum	Slow growth with short helix	Potential pathogen
M. simiae	Slow growth with short helix	Potential pathogen
M. triplex	Slow growth with short helix	Rare pathogen
M. triviale	Slow growth with short helix	Rare pathogen
M. asiaticum	Slow growth with long helix	Rare pathogen
M. avium subsp *avium*	Slow growth with long helix	Potential pathogen
M. avium subsp *paratuberculosis*	Slow growth with long helix	Not confirmed
M. bovis	Slow growth with long helix	Pathogen
M. celatum	Slow growth with long helix	Potential pathogen
M. conspicuum	Slow growth with long helix	Potential pathogen
M. cookii	Slow growth with long helix	Non pathogen
M. gastri	Slow growth with long helix	Rare pathogen
M. gordonae	Slow growth with long helix	Potential pathogen
M. haemophilum	Slow growth with long helix	Potential pathogen
M. hibernae	Slow growth with long helix	Non pathogen
M. intracellulare	Slow growth with long helix	Potential pathogen
M. kansasii	Slow growth with long helix	Potential pathogen
M. leprae	Slow growth with long helix	Pathogen
M. malmoense	Slow growth with long helix	Potential pathogen

Table 1.2.1.cont...

M. marinum	Slow growth with long helix	Potential pathogen
M. nonchromogenicum	Slow growth with long helix	Rare pathogen
M. scrofulaceum	Slow growth with long helix	Potential pathogen
M. shimodei	Slow growth with long helix	Rare pathogen
M. szulgai	Slow growth with long helix	Potential pathogen
M. terrae	Slow growth with long helix	Rare pathogen
M. tuberculosis	Slow growth with long helix	Pathogen
M. ulcerans	Slow growth with long helix	Potential pathogen
M. xenopi	Slow growth with long helix	Potential pathogen
M. aichiense	Fast growth with short helix	Non pathogen
M. alvei	Fast growth with short helix	Non pathogen
M. aurum	Fast growth with short helix	Non pathogen
M. austroafricanum	Fast growth with short helix	Non pathogen
M. chelonae	Fast growth with short helix	Potential pathogen
M. chitae	Fast growth with short helix	Non pathogen
M. chlorophenolicum	Fast growth with short helix	Non pathogen
M. chubuense	Fast growth with short helix	Non pathogen
M. confluentis	Fast growth with short helix	Non pathogen
M. diernhoferi	Fast growth with short helix	Non pathogen
M. duvalii	Fast growth with short helix	Non pathogen
M. fallax	Fast growth with short helix	Non pathogen
M. farcinogenes	Fast growth with short helix	Not confirmed
M. flavescens	Fast growth with short helix	Rare pathogen
M. fortuitum	Fast growth with short helix	Potential pathogen
M. gadium	Fast growth with short helix	Rare pathogen

As can be seen in Table 1.2.1., true pathogens are limited to the slow growers. Fast growing species are opportunistic pathogens, usually associated with wound infections, and rarely cause severe infections. One important sub-group of the mycobacteria, is the *Mycobacterium tuberculosis* complex, as it contains both *M. tuberculosis* and *M. bovis*, the causative agents of tuberculosis (Gordon *et al.*, 1999).

1.3. *The Mycobacterium tuberculosis* complex

The four members of the *M. tuberculosis* complex; *Mycobacterium tuberculosis, Mycobacterium bovis*, *Mycobacterium africanum* and *Mycobacterium microti* are the causative agents of tuberculosis in both humans and animals. These species have been shown to be very closely related through DNA-DNA hybridization, and have identical 16S rRNA gene sequences (Gordon *et al.*, 1999). Despite this genetic relatedness, the species show differences in certain phenotypic and genetic characteristics as shown in Table 1.3.1. The complex can be expanded to the *M. tuberculosis* clade, which contains the four species mentioned above and also *Mycobacterium ulcerans*, and *Mycobacterium marinum*.

Table 1.3.1. Discriminatory phenotypic and genetic characteristics of members of the *M. tuberculosis* complex (adapted from Niemann *et al.*, 2000)

Organism and group	Colony morphology	Growth in presence of TCH	Change of colour of bromcresol purple medium	PZA sensitivity
M. tuberculosis	Eugonic	+	+	S
M bovis				
Subsp. *bovis*	Dysgonic	-	-	R
Subsp. *capri*	Dysgonic	-	-	S
Subsp. *C*	Dysgonic	-	-	S
M. africanum				
Subtype I	Dysgonic	-	-	S
Subtype II	Dysgonic	+	-	S
M. microti				
Subtye Vole	ND	ND	ND	ND
Subtype Llama	ND	ND	ND	S

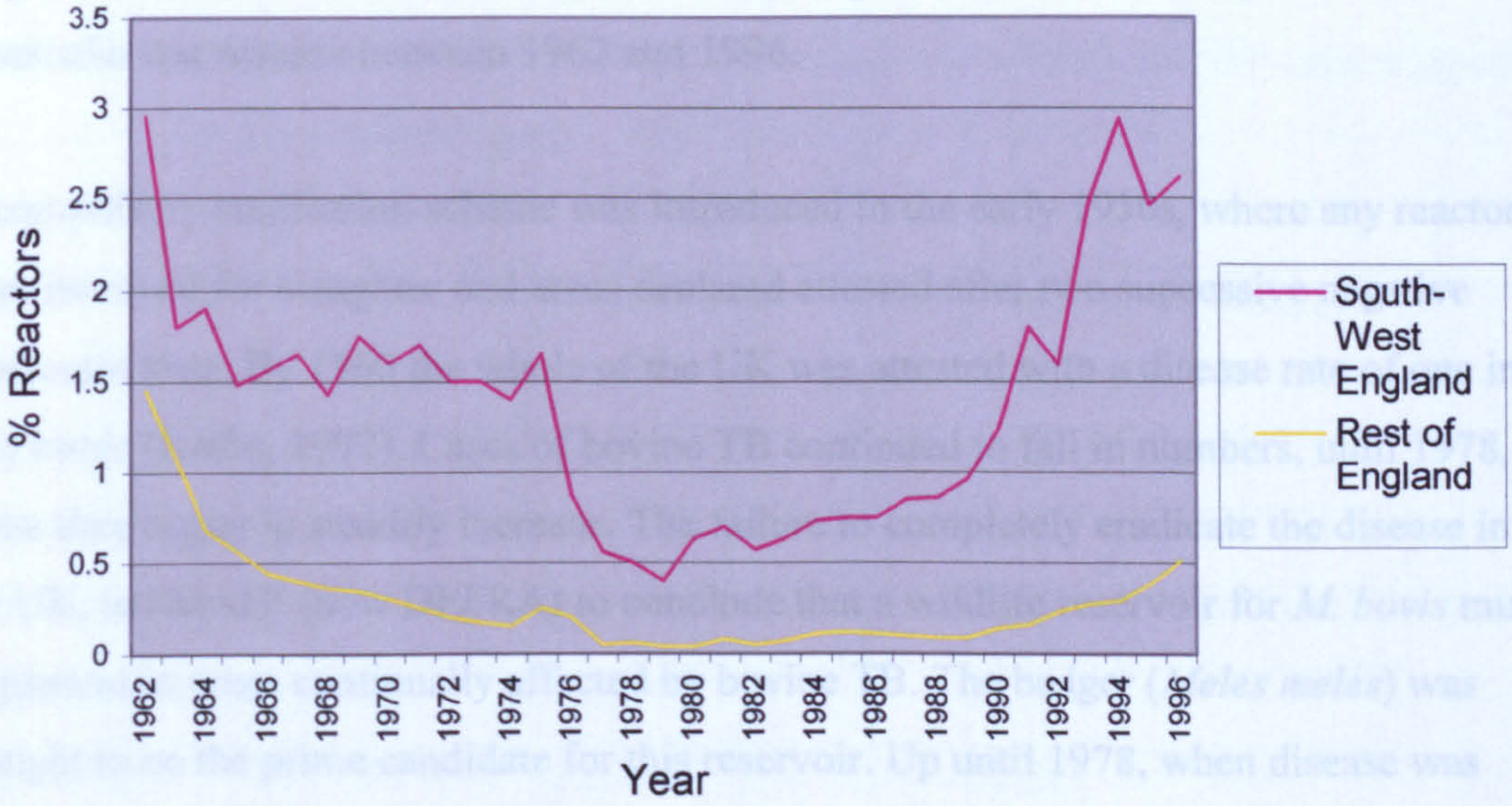
S, susceptible; R, resistant; +, positive test result; -, negative test result; ND, not determined.

Of the species, *M. tuberculosis* and *M. africanum* are most associated with human disease. It is believed that more than one third of the world's population are infected with *M. tuberculosis*, and that 60% of all pulmonary tuberculosis isolates from patients in Africa are *M. africanum* (Niemann *et al.*, 2000). *M. bovis* and *M. microti* are mainly associated with animal disease including cattle, badgers, goats and large cats for *M. bovis* and small rodents for *M. microti*. *M. bovis* is of particular concern in the UK and Ireland due the continuing rise in cases of bovine tuberculosis (Krebs, 1997).

1.4. *M. bovis* as a cause of tuberculosis

In recent years human infection with *M. bovis* has become less of a concern, with the main focus now placed on the role the organism plays in bovine tuberculosis. In the 1930s *M. bovis* was believed to be the cause of 6 % of all human TB cases in the UK (~2000 out of 33,000 deaths) (Hardie and Watson, 1992). The overall number of cases of TB in the UK has dropped significantly, with *M. bovis* cases dropping proportionally more so. Between 1993 and 1995 out of the 6,000 notified TB cases, only 1% were attributed to *M. bovis* infection. The main route of transmission of *M. bovis* to humans was believed to be though exposure to contaminated farm produce (in 1934, 40% of cows in UK dairy herds were infected), and changes in farming practices have led to the decrease in transmission. The transmission route for the majority of TB cases was thought to be through drinking un-pasteurised milk, from infected herds. In the 1930s, wide-scale pasteurization of milk products was introduced, and this dramatically decreased the risk of cattle to human transmission (Krebs, 1997). Currently un-pasteurised milk cannot be supplied from herds unless they have a "tuberculosis free" status. The introduction of a regular testing, and slaughter policy has led to a decrease in bovine TB cases, and subsequently human infections. Most cases of *M. bovis* infections in humans, are now caused by a direct exposure to infected cattle, or are reactivations of primary disease in older patients. The risk of contracting *M. bovis* in the UK has been calculated to be 1 in 2 million, compared to a 1 in 20, 000 risk of contracting *M. tuberculosis* caused TB (DEFRA, 2003)

Fig 1.1. Percentage of cattle herds with reactors. Source : DEFRA data.



Cases of bovine TB have reduced considerably in the UK since the 1930s, however they are once more on the increase (although not as high as previous levels). Fig 1.1 gives the proportion of all cattle herds in England that gave positive reactors (i.e. positive tuberculin test results) between 1962 and 1996.

A compulsory eradication scheme was introduced in the early 1950s, where any reactors were removed for slaughter and areas declared attested after two successive negative tuberculin tests. By 1960 the whole of the UK was attested with a disease rate of one in fifty herds (Krebs, 1997). Cases of bovine TB continued to fall in numbers, until 1978, when they began to steadily increase. The failure to completely eradicate the disease in the UK, led MAFF (now DEFRA) to conclude that a wildlife reservoir for *M. bovis* must be present in areas continually affected by bovine TB. The badger (*Meles meles*) was thought to be the prime candidate for this reservoir. Up until 1978, when disease was found in badgers and was attributed to herd breakdowns, The Badgers Act 1973 allowed licences to be issued for the gassing of badgers. In 1975, licences were limited to MAFF officials. By 1979 public opinion on the role badgers played in bovine TB changed and so a review head by Lord Zuckerman was initiated. While this review was carried out all gassing strategies were stopped. At this time the disease began to spread, particularly in the south west of England, and so it was ordered that the gassing strategy be resumed as soon as was feasibly possible. This strategy was replaced in 1982 by a "trap and shoot" policy, in the light of Zuckerman's report. Successive reports and acts in the following years led to badgers becoming highly protected species, with issuing of licences for badger removal limited further. Krebs (1997) reported that current badger protection acts, confer a degree of protection that is beyond that necessary to preserve their current distribution. The current opinion is that a link between bovine TB and badgers is present, although the exact nature of that link has not been confirmed.

1.5. Transmission of *M. bovis* to cattle

Although the badger has been implicated as the most likely candidate for spreading bovine TB, other factors have been investigated, including other wildlife reservoirs. Table 1.5.1 gives prevalence of *M. bovis* in infected wildlife, although it must be stated

that the number of individual animals investigated is extremely limited, and the data could vastly underestimate the actual occurrence of the organism.

Table 1.5.1. Prevalence of *M. bovis* infection in infected wildlife (MAFF annual reports on bovine tuberculosis in badgers).

Species	**Infection prevalence (%)**	**Number of animals sampled**
Mole (*Talpa europaea*)	1.21	166
Fox (*Vulpes vulpes*)	1.15	954
Mink (*Mustela vison*)	0.58	172
Rat (*Rattus norvegicus*)	1.12	412
Wild Deer	1.05	1,817
Ferret (*Mustela putorius furo*)	3.85	26
Badger (*Meles meles*)	4.05	21,731

Although the presence of *M. bovis* has been determined in these animals, it does not mean they are infectious. Only animals that actively shed *M. bovis* can transmit disease, and it is known that badgers in the late stages of disease often have severe systemic lesions from which shedding occurs (Clifton-Hadley and Wilesmith, 1991). Bacteria can be shed in sputum (lung infection), faeces (swallowed sputum), urine (kidney infection) and pus (infected bite wounds). Apart from badgers, only deer and ferrets have been found to actively shed *M. bovis* (Clifton-Hadley and Wilesmith, 1991).

The feeding habits of badgers have also led to the conclusion of a link between TB in badgers and cattle. Badgers prefer to feed on grazed pasture, and although direct contact between cattle and badgers is rare, cattle could come into contact with faeces and urine deposited by the badgers. There is also the possibility that these deposits could contaminate the pasture soil that would provide a reservoir for *M. bovis,* outside of an

animal host. This hypothesis is the main focus of research presented in this thesis and will discussed further.

There is evidence for a certain level of horizontal transmission between cattle, and infection of herds by cows brought in from other farms, or cattle stockists, are responsible for a small number of TB outbreaks. This form of transmission is rare, however, due to the nature of infection and detection of the disease in cattle. Reactivity to the tuberculin skin tests occurs only 50 days after infection, and so the majority of cases are detected before the disease becomes systemic in the animal. Cattle very rarely shed *M. bovis* into the environment. However it has been shown that during the early stages of infection, *M. bovis* can be found in the trachea, nasal mucus, and nasal pharynx of cattle, and so direct cattle to cattle transmission is possible (Krebs, 1997).

1.6 Diagnosis of disease and animal vaccination

Detection of the transmission routes of bovine TB is an important factor in the eradication of the disease. However, there are current initiatives to improve the mechanisms for detecting the disease, and also to investigate the possibility of vaccinating animals against *M. bovis*. Research presented in this thesis forms part of a current initiative by the Irish Government to vaccinate badgers with *M. bovis* BCG (Pasteur) with a view to lowering the prevalence of the organism in the badger populations of Southern Ireland.

1.6.1 Diagnosis

The currently used method of diagnosing cattle infected with *M. bovis* is that of the tuberculin skin test. This uses a purified protein derivative (PPD) from *M. bovis*, injected intradermally. In infected animals, this stimulates a local cellular immune response, which is detected three days after injection by measuring skin thickness. As non-specific reactions can be observed after cattle have come into contact with environmental mycobacteria, a second injection is made, containing PPD from *M. avium*. Three interpretations of the skin test can be applied to cattle. The first is a negative result, where no reaction is observed. Secondly a "standard interpretation" can be applied. This is

where the skin reaction is greater than 4 mm at the *M. bovis* PPD injection site compared to the *M. avium* PPD site. Reactions greater than 0 mm, but less than 4 mm are considered inconclusive and the animal is retested. Finally reactions greater than 4 mm or where visible tuberculous lesions have confirmed a reactor are classified as "severe interpretations". All animals which are classified as reactors are slaughtered, and *post mortem* examination carried out to determine the extent of the disease, and in most cases to type the causative strain (Krebs, 1997).

Where a herd breakdown occurs, the remaining animals are tested at 60-day intervals until two consecutive negative results are found in all animals. The herd is then tested again six and twelve months later. There is some concern that continuous testing leads animals being immunologically primed to the PPD. This would mean that the skin test reactions are to due previous tests and not due to an infection with *M. bovis*. Routine testing of herds for presence of *M. bovis* is carried out at regular intervals dependent on the location of the herd. Where the infection rate in the local area (usually the county) is greater than 1 %, all herds are tested annually. Where the infection rate is between 0.2 and 1 % the herds are tested once every two years, between 0.1 and 0.2 % once every three years, and below 0.1 %, once every four years. These rates and areas are governed by the European Union, and the legislation is particularly unclear at this point (DEFRA, 2003).

As can be seen the use of the tuberculin test is dependent on human interpretation of results. It does not give a clear yes or no answer as to the infection state of the animal, and relies on the skill of the person employed to carry out the test in order to give an accurate result. Despite this it appears that the specificity of the test is very high (above 99%). However, the sensitivity rate is of concern, with reports giving a rate between 75 and 95 % (Monaghan *et al.*, 1994). Indeed, the number of repeated herd breakdowns, possibly due to the initial infected animal not being discovered, has risen to a frequency to 16.5% of all breakdowns in 1996. There is also concern that the use of *M. avium* PPD may not be enough to distinguish between true infection and the reaction to exposure from environmental mycobacteria. In southern Ireland a proportion of tuberculin tests

gave false positive results, as determined by *post mortem* examination of cow carcasses. Certain species of mycobacteria, in particular *M. hiberniae*, have been shown to elicit extremely similar immune responses in animal hosts to those elicited by *M. bovis*. It is thought that these species are responsible for the high number of false positives recorded in Ireland (approx. 25 % of all positives) (Gormley, personal communication).

To overcome this new detection methods are being developed. These include the refining of the PPD to make it more specific in detecting *M. bovis*. Certain antigenic proteins are being targeted including MPB70, which is secreted by actively growing *M. bovis* cells, and is only found in members of the *M. tuberculosis* complex, and MPB64 and ESAT6, again specific for the complex (Li *et al.* 1993; Alito *et al.*, 1999). Replacing the PPD with a single antigen, or a cocktail of these antigenic proteins would significantly reduce the number of false positives. Other alternatives include laboratory analysis of samples taken from presumptive infected cattle, in particular blood testing. This would involve the culture of blood lymphocytes with antigens derived from *M. bovis* and measuring the proliferation of T lymphocytes (Gormley *et al.*, 1999). A large-scale field trial of this in Australia resulted in sensitivity and specificity values of 93.6 and 96.3 % respectively (Krebs, 1997). The advantage of this system is that it can give a definitive positive or negative result of infection, without the need for human interpretation. One drawback in this approach is the cost involved. PPD testing is relatively cheap, and the increased manpower, and laboratory time needed for the lymphocyte assay precludes its use at the current time.

A final problem with using PPD in detecting bovine TB is its use on vaccinated animals. Strategies for vaccinating animals rely on the ability to differentiate between infected and vaccinated animals. PPD testing cannot differentiate between these, and so a cheap and reliable detection system must be in place before full-scale vaccination of animals can occur. (Pollock *et al.*, 2003).

1.6.2 Vaccination of animals

Currently two strategies for the vaccination of animals involved in bovine TB are in place. One is the vaccination of cattle (DEFRA) and the second is the vaccination of badgers (Irish government). The use of vaccines was reviewed by the World Health organization in 1994 and two classes were identified; vaccines with defined efficacy which could be implemented within 4 to 6 years, and vaccines currently in development, with a time frame of use within 15 years. Candidates currently include the human TB vaccine strain *M. bovis* BCG, crude protein derivatives of *M. bovis*, DNA vaccines and environmental mycobacteria. Two trials of using BCG as a vaccine candidate have been carried out. The first by Buddle *et al.* (2003) recorded varying efficacies of protection, whereas the second showed a huge drop in the number of animals with lung lesions, although the total infection rate was still high (11 out 27 animals) (Buddle *et al.*, 1995). The use of BCG is also affected by the presence of environmental mycobacteria, which have been shown to interfere with the protective immunity given to animal by the vaccine.

The Irish government is investigating the use of *M. bovis* BCG in the vaccination of badgers in problem areas of Ireland. Efficacy is of concern, as is the actual uptake of the vaccine by the animals. Trapping and injection of the badgers is not currently feasible, so the vaccine is to be placed in feed baits in the areas of badger sets. The target areas of vaccination are also being carefully studied. The currently opinion is that areas with highly infected badgers will not be targeted, instead, animals there will be trapped and removed, and sets decontaminated. Areas where infection of badgers is low or non-existent will be target for vaccination; in an effort to reduce the number of infected badgers across Ireland.

One problem with using BCG as a vaccine in feed bait is the risk that the organisms could survive and proliferate in the environment. TB problem areas tend to have badger sets in close proximity to pasture land, and there is a risk that if the organisms remain viable for significant periods of time in soil, cattle could come into contact with them. If these cattle should take up the organism, they would become reactors to the PPD skin

test, and have to be removed, although in effect they would be vaccinate animals. Research resented in this thesis sought to instigate the survival and levels of viability of *M. bovis* BCG under defined environmental conditions.

1.7. *M. bovis* in the environment

As stated previously there is a concern as to the survival of *M. bovis* BCG in the environment if the current strategy of feed baiting is carried out. Also of concern is the survival of *M. bovis* itself in the environment of farms where badgers are actively shedding the bacteria. All research previously carried out has used viable cell culture methods to determine cell numbers in soil or faecal samples. There are problems associated with this approach, and these will be discussed further in **Chapters 3** and **4.** In work carried out by MAFF (1979), *M. bovis* was shown to survive for up to 28 days on pasture land when suspended in badger urine, 70 days in bronchial pus and 28 days in faeces. The results of this research however must be questioned as it is extremely unlikely that in the environment *M. bovis* cells would remain suspended in the excretions and would be effected by rainfall, possibly percolating into the soil sub-surface and beyond. Krebs (1997) reported that the soil environment should be considered as a possible source of infection, and molecular analyis of these soils should be carried out in order to quantify this risk. Despite this recommendation presented in 1997, no research on the survival of *M. bovis* in soil has been carried out since this date. Research presented in this thesis is the first carried out to determine the presence and survival of *M. bovis* and *M. bovis* BCG in soil using molecular detection techniques.

One factor in the ability of *M. bovis* to survive in the environment may be the ability of certain mycobacteria to enter a dormant state. This has been particularly well studied in *M. tuberculosis*, where after treatment with antibiotics for infections in humans, the infection appears to have been cleared, with no viable cells being cultured from affected tissue samples. These particular cases of disease can become reactivated if the treatment is stopped too early, and also can reactivate many years after the initial infection. This phenomenon led to the hypothesis that these cells could enter a highly resistant dormant state inside the host. Using an *in vitro* model, Wayne and Lin (1982) found that in a

microaerophilic sedimented layer of a culture of *M. tuberculosis*, non-dividing yet viable cells were present. This dormant state was believed to be characterised by a down regulation of metabolic processes and a shift in to the glyoxylate cycle and has become known as the Wayne dormancy model. These cells became highly resistant to the antibiotics isoniazid and rifampin, but became susceptible to the drug metronidazole, which is used in the treatment of infections caused by anaerobic bacteria. It is thought that this down-regulation, is due to an adaptation by the cell to survive in increasingly anoxic environments, in the case of *M. tuberculosis*, its survival within granulomas in the animal host. The cell wall has been shown to have an important role in this dormant state. Cunnigham and Spreadbury (1998) showed that the cell wall of *M. bovis* BCG thickens substantially during oxygen deprivation, while the cells overall become thinner and smaller in shape. This was seen more notably in *M. tuberculosis*. An up-regulation in the production of an α-crystallin protein was also observed. This protein can prevent the thermal aggregation of proteins, without the loss of enzymatic activity. This up-regulation appears to be under the control of the sigma factor SigF. This has been shown to control the expression of many genes involved in metabolic activity and also in nutrient acquisition i.e. siderophores (Boon *et al.*, 2001). This could possibly mean that oxygen deprivation is not the only trigger for dormancy, and that other factors, such as nutrient deprivation and cold shock may form an important part in the onset of this stringent response. *M. tuberculosis* contains genes similar to those found in sporulating bacteria, in particular the gene encoding for the 21 kDa spore protein of *Stigmatella aurantiaca*, and *whiB* sporulation factor of *Streptomyces* species (Hutter and Dick, 1999). Although mycobacteria are non-spore forming, it is believed that there may be a substantial link between sporulation and dormancy, and that they may possess similar genetic backgrounds. If this is the case then it is feasible that as nutrient levels and oxygen levels in soil would be low, then dormancy could be triggered in mycobacteria present in a soil environment.

1.8. Aims of the research presented in this thesis

The aim of this research was to determine the extent of survival of *M. bovis* and *M. bovis* BCG in the soil environment both in the laboratory and in the field. The use of both cell culture and molecular detection methods would be investigated, with the use of DNA and RNA extraction followed by analysis investigated as a way of significantly reducing the time needed to positively identify *M. bovis* in soil.

Secondly to determine the detectable diversity of the mycobacteria population in soil from a farm in southern Ireland in areas of infection and non-infection with bovine TB.

Chapter 2: Materials and methods

2.1. Buffers and solutions

All chemicals described were of AnalaR grade and were supplied by Sigma-Aldrich Co. Ltd or BDH unless otherwise stated.

Loading dye (6X): 0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% (w/v) glycerol.

TAE (50X): 10 M Tris-acetate (pH7.0), 250 mM EDTA (pH8.0).

TE buffer (pH8.0) (10X): 100 mM Tris-HCl (pH8.0), 10 mM EDTA.

2.2. Media

LB (Luria Bertani) medium.

Bacto tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Distilled water	1000 ml

For LB agar 15 g of Bacto-agar was added. The medium was sterilised by autoclaving at 121°C for 20 min.

SOB medium:

Bacto tryptone	20 g
Yeast extract	5 g
NaCl	0.5 g
KCl	2.5 mM (pH7.0).
Distilled Water	1000 ml

The medium was sterilised by autoclaving at 121°C for 20 min. The solution was allowed to cool before the addition of 5 ml of sterile 2 M $MgCl_2$.

SOC medium.

SOB medium supplemented with glucose to a final concentration of 20 mM.

GYM *Streptomyces* medium

Glucose	4.0 g
Yeast extract	4.0 g
Malt extract	10.0 g
$CaCO_3$	2.0 g
Agar	12.0 g
Distilled water	1000.0 ml

pH was adjusted to 7.2 with KOH before adding agar. The medium was sterilised by autoclaving at 121°C for 20 min.

Glycerol-soil extract medium

Peptone	5.0 g
Beef extract	3.0 g
Glycerol	20.0 g
Soil extract	150.0 ml
Distilled water	850.0 ml
Agar	15.0 g

pH was adjusted to 7.0. Soil extract was prepared by sieving air-dried garden soil through a coarse sieve and autoclaving 400 g with 960 ml of distilled water at 121°C for one hour. After the mixture was cooled, the supernatant was decanted, filtered through paper, autoclaved in 200 ml quantities, and stored at room temperature until cleared by sedimentation. The medium was sterilised by autoclaving at 121°C for 20 min.

***Micromonospora megalomicea* medium**

Glucose	10.0 g
Soluble starch	20.0 g
Yeast extract	5.0 g
Casitone	5.0 g
$CaCO_3$	1.0 g
Agar	15.0 g
Distilled water	1000.0 ml

The medium was sterilised by autoclaving at 121°C for 20 minutes.

Middlebrook 7H9 broth

4 g of dried 7H9 powder (Difco) was dissolved in 895 ml of distilled water and 5 ml glycerol.

Middlebrook 7H10 agar

19 g of dried 7H10 powder (Difco) was dissolved in 895 ml of distilled water and 5 ml glycerol.

Both Middlebrook media were autoclaved at 121°C for 10 min. After cooling 100 ml of OADC enrichment was added to 900ml of either Middlebrook media.

OADC Enrichment

BSA	10 g
Dextrose	5 g
NaCl	1.75 g
Catalase	0.5 g
Oleic acid	0.25 ml
Distilled water	100 ml

After mixing, enrichment was filter sterilised through a 0.2 µm disc filter (Acrodisc, Pall Life Sciences, Portsmouth, UK or Sartorius, Goettingen, Germany).

2.3. Bacterial species

Species used throughout this research are listed in Table 2.3.1.

Table 2.3.1. List of bacterial species and strains used.

Bacterial Species	**Strain/Identification Number**
Actinomaudra malachitiea	DSM 43462*
Actinoplanes auranticolor	DSM 43031*
Actinosynnema mirum	JCM 3225**
Arthrobacter oxydans	JCM 2521**
Brevidobacterium flavum	JCM 2180**
Escherichia coli	TOPO F` (Invitrogen)
Micromonospora echinospora	ATCC 15838***
Micromonospora rosea	ATCC 21946***
Mycobacterium abscessus	****
Mycobacterium agri	****
Mycobacterium aichiense	****
Mycobacterium aurum	ATCC 23366***
Mycobacterium bovis BCG	Pasteur
Mycobacterium chitae	****
Mycobacterium chubuense	****
Mycobacterium duvalii	****
Mycobacterium fortuitum	ATCC 14468***
Mycobacterium fortuitum	Soil isolate
Mycobacterium gadium	****
Mycobacterium genavense	****
Mycobacterium gilvum	****
Mycobacterium gordonae	ATCC 14470***
Mycobacterium marinum	****

Table 2.3.1 cont...

Mycobacterium neoaurum	****
Mycobacterium nonchromogenicum	ATCC 19530***
Mycobacterium obuense	****
Mycobacterium parafortuitum	****
Mycobacterium peregrinum	****
Mycobacterium phlei	ATCC 354***
Mycobacterium senagalense	****
Mycobacterium smegmatis	ATCC 13578***
Mycobacterium terrae	ATCC 15753***
Mycobacterium thermoresistible	****
Nocardia brevicatena	ATCC 15333***
Amycolatopsis petroleophila	ATCC 15776***
Planobispora rosea	JCM 3166**
Planomonospora venezualensis	ATCC 23865***
Rhodococcus coprophilus	ATCC 184***
Rhodococcus luteus	ATCC 35014***
Spirilospora albida	-
Streptomyces coelicolor	A3(2)
Streptomyces lividans	ATCC 19844***
Streptomyces rochei	-
Streptomyces violaceus	ATCC 15888***
Streptosparangium roeseum	-
Streptoverticillium griseocarneum	ATCC 23934***

* Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH Mascheroder Weg 1b, 38124 Braunschweig, Germany

** Japan Collection of Microorganisms, RIKEN (The Institute of Physical and Chemical Research), 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

*** American Type Culture Collection (ATCC), P.O. Box 1549, Manassas, VA 20108, USA

****Supplied by John Magee, Regional Centre for Mycobacteriology, Newcastle, UK

2.4. Soil sampling

Soil samples were taken from a farm with a history of bovine tuberculosis located in Ireland (OSI (Ordinance Survey Ireland) reference: O1089) in April 2000 and in November 2002. The farm had undergone a herd breakdown approx. 4 months prior to sampling. 11x 1 m^2 sampling sites were chosen, soil from entrances to badger sets, soil from pasture-land on which the infected cattle grazed, and adjoining fields. These sites were designated as BS1 and BS2 (Badger set soil), and A1 to A9 for the remaining sampling sites. Ten x 10 cm cores were taken from each site. As a comparison, soil was also taken from a site close to Warwick University, UK. This site had no previously known recent history of use by cattle. Several other soils were also used from different geographical regions, including soils from Greece, Italy, Cuba, the Cotswolds and North Wales (UK).

2.5. Cell extraction from soil

2.5.1. Decontamination method

Mycobacterium strains were extracted from soil using the following method; 10 g of soil were added to 10 ml of quarter strength Ringer's solution in a sterile Universal tube. Tubes were shaken for 10 min on a Griffin shaker followed by addition of NaOH to give a concentration of 3% and were then incubated for 30 min at room temperature. The soil solutions were mixed, then 100 µl were plated onto Middlebrook 7H10 agar plates containing cycloheximide (1 mg/ml) and nystatin (1 mg/ml). Plates were incubated in gas permeable bags for 8 weeks at 30°C.

2.5.2. Non decontamination method.

Mycobacterium strains were extracted from soil using the following method. 10 g of soil were added to 10 ml of quarter strength Ringer's solution in a sterile Universal tube. Tubes were shaken for 10 min on a Griffin shaker then 100 µl amounts were plated onto Middlebrook 7H10 agar plates. Plates were incubated in gas-permeable bags for 8 weeks at 30°C.

2.6. Nucleic acid extraction

2.6.1. Chromosomal DNA extraction from cells

With cells grown in liquid media, 2 ml of a stationary phase culture was centrifuged at 13,000 rpm for 5 min. Supernatant was removed and pellet resuspended in 500 µl of TE buffer. For cells cultured on solid media, a loop of biomass was added to 500 µl of TE buffer and vortexed to mix. For both types the extraction proceeded as follows; 2 mg of lysozyme were added to the prepared culture, vortexed to dissolve and then incubated for 1 h at 30°C. 120 µl of 0.5 M EDTA was added and incubated for a further 45 min at 30 °C. After incubation 124 µl of Proteinase K (1mg/ml) and 70µl of SDS (10%) was added followed by an incubation step of 20 min at 65°C. Addition of 300 µl of sodium perchlorate (5M) was followed by an incubation step of 20 min at 30°C.

Samples were then cooled on ice and then mixed with an equal volume of 1:1 phenol/chloroform: isoamylalcohol (24:1), followed by centrifugation at 13,000 rpm for 15 min. The aqueous phase was removed and the phenol/chloroform : isoamylalcohol extraction repeated on this phase. The aqueous phase was removed again and added to an equal volume of chloroform. Samples were centrifuged at 13,000 rpm and the aqueous phase removed. Two volumes of ethanol was added to the aqueous phase and the preparation placed at -20°C overnight, then centrifuged at 13,000 rpm for 15 min. Supernatant was removed and the pellet resuspended in 100 µl of TE buffer.

2.6.2. Community DNA extraction from soil (method 1)

1 g of soil, 1 g of 0.1 mm glass beads and 5 ml of TE buffer were place in a sterile glass bead-beating tube. Bead-beating was then carried out for 5 min with CO_2 cooling. 200 µl of lysozyme (100 mg/ml), 100 µl SDS (10%) and 75 µl Proteinase K solution (25 mg/ml) was added and the samples placed at 37 °C for 45 min. Following a centrifugation step at 4,000 rpm for 15 min, the supernatant was removed and mixed with 5 ml of potassium acetate (8M), and placed on ice for 15 min. After a further centrifugation at 15,000 rpm for 30 min, the supernatant was removed and added to 10 ml PEG 6000 and 2 ml of NaCl (5M), incubated overnight at –4 °C, then centrifuged at 15,000 rpm for 30 min. The

supernatant was then passed through either a Chelex, Sephadex, or a Sepharose 4B column.

2.6.3. Community DNA extraction from soil (method 2)

DNA was extracted using the Mobio Soil Microbial DNA extraction kit as per the manufacturer's instructions (Mobio Laboratories Inc. Solana Beach, CA).

2.6.4. RNA extraction from cells

For cells grown in liquid media, 10 ml of a stationary phase culture were centrifuged at 13,000 rpm for 5 min. The supernatant was removed and the pellet re-suspended in 500 µl of TE buffer. For cells cultured on solid media, a loop of biomass was added to 500 µl of TE buffer and vortexed to mix. RNA was then extracted using the Mobio microbial RNA extraction kit, as per the manufacturer's instructions (Mobio Laboratories Inc. Solana Beach, CA).

2.6.5. Community RNA extraction from soil (method 1)

1 g of soil was mixed with 1g of 0.1mm glass beads, and 1ml of DEPC water in a sterile microcentrifuge tube. This was then vortexed for 10 min to lyse the cells. 1 ml of Catrimox-14 (IOWA biosciences) was added and the tube vortexed for a further 1 min. Samples were then centrifuged at 13,000 x rpm for 5 minutes at 14°C. The supernatant was removed and 0.5 ml of 0.5 ml of 2M LiCl was added, vortexed for 1 min, and centrifuged for 5 min at 13,000 rpm. The supernatant was removed and the pellet washed with 70% ethanol, dried, and then resuspended in 500 µl of DEPC water. RNA samples were treated with RNase free DNAse (Qiagen) as per the manufacturer's instructions. Treated RNA samples were extracted once in chloroform and finally precipitated with ethanol. Finally they were resuspended in 100 ul of DEPC-treated water.

2.6.6. Community RNA Extraction from soil (Method 2)

RNA was extracted from soil using the Mobio microbial RNA extraction kit, with a slightly modified method. The contents of one extraction tube were added to 1 g of soil in a sterile microcentrifuge tube. The method then continued as per the manufacturer's instructions.

2.6.7. Agarose gel electrophoresis

Nucleic acids were visualised on 1% agarose gels. 1 g of agarose was added to 100 ml of 1 x TAE buffer and heated in a microwave oven until dissolved. After cooling, and addition of 6 µl of ethidium bromide, the melted agarose was poured into a gel tray, a comb added, and allowed to set. 10 µl of nucleic acid sample were mixed with 2 µl 6 x gel loading dye and loaded onto the gel. Gels were electrophoresed at 100 V for 1 h. Nucleic acids were visualised on a UV transilluminator (UV products Inc, USA.). Images of the DNA fragments were taken by a UVI tech gel documentation system.

2.7. PCR analysis

2.7.1. PCR primers

The PCR primers targeted to the *M. bovis* group of organisms are shown in Table 2.7.1. Primers designed to target *Mycobacterium* spp. 16S rRNA encoding genes were generated, as shown in Table 2.7.2. JSY16SF/R were designed to target the whole *Mycobacterium* genus, where as JSY16SslowF/R were designed to target the slow growing group of the *Mycobacterium*. Genus. JSY16SslowF was based around the sequence insert at bases 402 to 431 found only in slow-growing species.

Table 2.7.1 Nucleotide sequences of PCR primers targeted to three genes specific to the *M. bovis* group of the mycobacteria.

Primer Name	Sequence	Amplified product length	Reference
MPB64 Forward (MPB64F)	cag gca tcg tcg tca gca gc	543	(Gormley *et al.* 1999)
MPB64 Reverse (MPB64R)	gtg att ggc ttg cga tag gc		(Gormley *et al.* 1999)
Esat-6 Forward (ESAT6F)	aca tga cag agc agc agt gg	413	(Gormley *et al.* 1999)
Eat-6 Reverse (ESAT6R)	tga caa cct ctc aga gtg cg		(Gormley *et al.* 1999)
MPB70 Forward (MPB70F)	gaa caa tcc gga gtt gac aa	471	(Gormley *et al.* 1999)
MPB70 Reverse (MPB70R)	agc acg ctg tca atc atg ta		(Gormley *et al.* 1999)

Table 2.7.2. Nucleotide sequences of PCR primers targeted to the 16S rRNA encoding genes of the mycobacteria.

Primer Target and Name	Sequence	Amplified Product length	Reference
16S rRNA Forward primer (JSY16SF)	tgg gaa act ggg aaa ctg ggt cta ata	468 – 465	This thesis
16S rRNA Reverse primer (JSY16SR)	ccc gca cgc cca agt taa gct gtg ag		This thesis
16S rRNA Slow Forward Primer (JSY16SslowF)	cga cga agg tcc ggg ttc tct cgg att gac	605	This thesis
16S rRNA Slow Reverse Primer (JSY16SslowR)	gcc atg cac cac ctg cac aca ggc cca c		This thesis

2.7.2. PCR conditions

A master mix was created for all primer sets as set out in Table 2.7.3

Table 2.7.3 Ingredients for PCR mastermix, giving each addition, amount and concentration.

Ingredient	**Amount added**	**Concentration**
$MgCl_2$	100 µl	50 µM
Invitrogen PCR reaction buffer	100 µl	-
BSA	100 µl	100 µg/ml
dNTPs	40 µl	100 µM
Forward primer	20 µl	0.1 µg/µl
Reverse primer	20 µl	0.1 µg/µl
Distilled H_20	510 µl	-

For each PCR reaction 45 µl of master mix was added to 1 µl DNA and 0.3 µl of *Taq* DNA polymerase (Invitogen). The PCR was then carried out under the following cycle conditions for each primer set;

MPB64, MPB70, Esat-6

95°C for 3 min, followed by 30 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 60 s, finally a single extension step of 72°C for 5 min.

JSY16SF/R

94°C for 5 min, followed by 35 cycles of 94^0C, 55^0C for 1 min, 65°C for 1 min, and finally a single extension step of 65^0C for 5 min.

JSY16SslowF/R

94°C for 5 min, followed by 35 cycles of 94^0C, 57^0C for 1 min, 67°C for 1 min, and finally a single extension step of 67^0C for 5 min.

All PCR products were visualized on 1 % agarose gels in TAE buffer, stained with ethidium bromide as above.

2.7.3 RT PCR

RT-PCR was carried out using Superscript II (Invitrogen) as per the manufacturer's instructions.

2.8. Quantification of PCR products

For all PCR quantitation, primer sets MPB64 and MPB70 were used.

2.8.1. Linearity of PCR

To determine when the PCR reaction remained linear for all primer sets, a PCR was carried out using 35 replicates. One replicate was removed after each PCR cycle and immediately stored at –20°C. These replicates were then visualized on a 1% agarose gel, and images of the DNA fragments taken by a UVI tech gel documentation system, saved in an uncompressed TIFF format. This image was then analysed for pixel intensity of each band using the TotalLab 1d gel program.

2.8.2. Creation of calibration curve

A set of standards was created using a dilution series of *M. bovis* BCG (Pasteur) from 10^9 to 10^1 cells per ml. Each dilution was inoculated into 1 g of sterile Warwick soil, the soil was then dried and the DNA extracted. The PCR was carried out and a calibration curve created using TotalLab 1d gel analysis program to determine pixel intensity of each band. These DNA standards were used in every PCR experiment and electrophoresed alongside unknown products.

2.8.3. Efficacy of quantitation

To determine the efficacy of the quantitation, blind testing was carried out. A culture of *M. bovis* BCG was randomly diluted into 2 ml aliquots. 1 ml of each dilution was added

to 1 g of sterile Warwick soil, DNA was then extracted and quantification carried out. The remaining aliquot was used to count cell content with a haemocytometer. The results were then compared.

2.8.4. Quantitation of PCR products from community DNA

PCR was carried out on soil community DNA using the method described in **2.6.3.** with only a 28 cycle reaction used. PCR was also carried out on the *M. bovis* BCG DNA dilution standards at the same time. All products were visualised on the same agarose gel and quantitated using the TotalLab 1d gel analysis program to measure pixel intensity of bands.

2.9. Microcosm experiments

For experiments requiring sterilised soil, samples were dried and sieved using a 4 mm mesh, then autoclaved at 121^{0}C for one hour and this was repeated after 24 h.

For all destructive microcosm experiments, 1g of either sterile or non-sterile soil was placed in a sterile Universal tube. Microcosms were inoculated with 10^8 *M. bovis* BCG cells, wetted to give a water content of 15 %, and incubated at room temperature unless otherwise stated. To monitor cell number over time both plate counts and PCR analysis were carried out for sterile soil microcosms, with PCR analysis only for non-sterile microcosms. Analysis was carried out at the following time points for all microcosms: Day 0, 1, 2, 3, 4, 5, 6, 7, 10, 13, 16, 20, 25, 30, 60, then 4, 5, 6, 8, 10, 12, 15, 18 months after initial inoculation. All experiments were carried out in triplicate.

The effects of several factors on survival of *M. bovis* BCG was investigated, Table 2.9.1 gives the environmental conditions investigated.

Table 2.9.1 Conditions of each microcosm experiment showing variations of temperature, soil type, and water content

Experiment	Temperature (°C)	Water content (%)	Inoculum type	Soil type
Suvival of *M. bovis* BCG	22	15%	Live cells	Sterile + Non-sterile
Effect of soil water content	22	5, 10, 15, 20, 30, 40	Live cells	Sterile + Non-sterile
Effect of Temperature	4, 15, 25, 37	15	Live cells	Sterile + Non-sterile

2.10. Turnover of DNA in soil

Microcosms were set up as in 2.12. DNA was inoculated into non-sterile soil microcosms in four states: as live cells, in dead but intact cells, in lysed cells, and as free DNA. Dead intact cells, were prepared by treating *M. bovis* BCG with UV light for 5 min. Lysed cells were created by heating *M. bovis* BCG at 100°C for 10 min. Lysis and non-lysis was observed using light microscopy and plate count methods. DNA was extracted as in 2.6.3. 10^8 cells were inoculated into each microcosm or 10^8 genome equivalents in the case of free DNA. Microcosms were incubated at both 10°C and 25°C, with samples taken every day for 21 days. DNA was then re-extracted from these samples and PCR carried out using *mpb70* and *mpb64* targeted primers. Products were visualised and subsequently quantitated using the method describe in **2.8.4.**

2.11. Cloning of PCR products

2.11.1. Cloning

Products of PCR reactions were ligated into TA cloning vectors (Invitrogen) as per the manufacturer's instructions. Chemically competent TOPO *E. coli* cells (Invitrogen) were transformed with the resulting plasmids and subsequently plated out onto LB agar plates

containing 100 µg/ml ampicillin, x-gal and IPTG. Colonies were picked for plasmid sequencing using blue/white selection. All steps carried out as per the manufacturer's instructions.

2.11.2. Plasmid preparation

For extraction of plasmid DNA, transformed *E. coli* colonies were cultured in LB broth, containing 100 µg/ml ampicillin, overnight at 37 °C. Plasmid DNA was then extracted using Qiagen Mini-prep plasmid purification kits as per the manufacturer's instructions.

2.12. Sequencing

2.12.1. Sequencing reactions with Big-Dye™

Sequencing reactions for the departmental automated sequencing service were performed in an Eppendorf PCR machine in a final volume of 10 µl. For sequencing plasmid DNA, 0.5 µg of template was used with 10 pmol of primer and 4 µl of Big Dye ™ (PE Applied Biosystems) enzyme mix. The PCR protocol consisted of 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. Products were precipitated by addition of 1 µl of 3 M sodium acetate pH5.2 and 25 µl of ethanol and placed at -20°C for at least 30 minutes. The samples were centrifuged at 13,000 rpm for 30 minutes, the supernatant was discarded and the pellet was washed with 100 µl of 70% ethanol and allowed to dry. The dried pellet was resuspended in loading buffer and electrophoresed on a polyacrylamide sequencing gel in an Applied Biosystems ABI 377 sequencer.

2.12.2. Sequence analysis

Sequences were analysed using Chromas V 2.22 (http://www.technelysium.com.au). Vector sequences were removed and where possible ambiguous bases were corrected. Resulting sequences were imported into Blast–N (http://www.ncbi.nlm.nih.gov/BLAST) to find the most closely related sequence in the GenBank database (http://www.ncbi.nlm.nih.gov). When two or more sequences showed greater than 99%

identity to a particular sequence in the database, they were counted as replicates in further analyses.

2.13. Phlyogenetic analysis of sequences

2.13.1. Alignments

Sequences were aligned using ClustalW (http://www.ebi.ac.uk/clustalw) and the resulting alignments, along with relevant sequences from Genbank, were imported into Bioedit v5.0.9 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Unidentified bases were removed as well as overhangs at each end to give sequences of equal length with the same start position. Gaps were added or removed to ensure the sequences were aligned along their entire length. Final alignments were saved as Phylip format files.

2.13.2. Construction of phylogenetic trees

Phylogenetic trees were generated using the Phylip suite of programs v3.5 (http://evolution.genetics.washington.edu/phylip.html). Firstly 100 replicates of input alignments were created using Seqboot. The resultant file was opened in either of the following.

1) Dnadist, using the Kimura 2-parameter method with a transition/transversion ratio of 2 to give a distance based matrix.
2) Dnamlk with a transition/transversion ratio of 2 to give a maximum likelihood matrix.

Dnadist output files were opened in Neighbor to give a neighbour-joining tree. This output and the output from Dnamlk gave 100 replicated trees that were condensed to one representative tree using Consense. Resulting trees were visualised using Treeview v1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

2.14. Statistical analysis

All experiments were carried out in triplicate unless otherwise stated, and results graphically represented with average values and standard deviations plotted. For analysis of 16S rRNA gene sequence diversity, EstimateS was used (http://viceroy.eeb.uconn.edu/EstimateS). This software estimates species richness and shared species content of samples tested. For diversity statistics, outputs are in the form of Sobs (Number of species observed in the pooled samples), ACE (Abundance-based Coverage Estimator of species richness) (Chazdon *et al.*, 1998), Jack1 (First-order Jackknife richness estimator) and Bootstrap (Bootstrap richness estimator) (Smith & van Belle, 1984), MMruns and MMmeans (Michaelis-Menten richness estimator: estimators averaged over randomizations (runs) and estimators computed once for mean species accumulation curve (means))(Raaijmakers, 1987). Shared Species estimators are presented as number of observed species and the number of estimated species based on comparisons across all samples. Finally Coleman richness expectation curves were created which estimate sample species richness from the pooled total species richness, based on all species actually discovered, whereas the other statistics estimate total species richness from samples, including species not discovered in any sample (Coleman, 1981; Coleman *et al.* 1982).

Chapter 3: Survival of *Mycobacterium bovis* BCG (Pasteur) in the Environment

3.1 Introduction

The tuberculosis vaccine *Mycobacterium bovis* BCG (bacillus Calmette-Guerin) has been used as a human vaccine to prevent infection by *Mycobacterium tuberculosis* since 1928 (Lowrie 1999), however, it also provides protection against the related organism *M. bovis*, which although it can cause tuberculosis, is not predominantly associated with human disease at the current time (Krebs 1997). This organism is, however, a cause for concern in that it is the causative agent of bovine tuberculosis, which is a severe problem in both the UK and Ireland.

The Irish Department of Agriculture and Food has set in place a system for badger vaccination in order to combat the high incidence of bovine TB in Ireland (Hughes *et al.* 1996). This initiative is currently not being employed in the UK, with DEFRA investigating the vaccination of cattle as an alternative measure. Both these approaches have advantages and disadvantages associated with them. Vaccination of cattle would be carried out through sub-dermal injection and therefore it would be guaranteed that all targeted animals would be processed. This would not be the case with badger vaccination, as the vaccine is to be placed in feed baits placed at set entrances, there would be no guarantee that all badgers in these sets would take up the vaccine. In both initiatives the efficacy of *M. bovis* BCG as a vaccine is of concern (Suazo *et al.*, 2003). An individual's response to the vaccine can be lowered due to the contact of the animal with environmental mycobacteria (Stanford *et al.*, 1981; Fine 1994; Buddle *et al.*, 2002). Both badgers and cattle would have a high contact rate with these organisms due to their feeding lifestyles. The vaccination of badgers would have to be carried out in conjunction with a testing and slaughter policy, as there is little point in vaccinating heavily infected animals. Sets would have to be investigated for presence of infected animals, these animals slaughtered and only non-infected sets targeted for vaccination. The vaccination of cattle (currently the only vaccine widely available is *M. bovis* BCG) would mean an individual animal, once vaccinated, would give a false positive result when tested for tuberculosis using the current PPD skin test (Wards *et al.*, 2000). Under current European law, cattle from these herds would lose their Officially Tuberculosis Free status, be placed under movement restrictions and would not be able to produce untreated milk for

sale direct to the public (DEFRA, 2003). Although this problem is not immediately of concern in the badger vaccination method, it could indirectly affect the feasibility. In small-scale field trials badgers were vaccinated with *M. bovis* BCG supplied in feed bait (Southey *et al.*, 2001; Southey *et al.* 2002), and there was concern as to the longevity and dissemination of the vaccine once in the environment, with worries of cattle coming into contact with the vaccine and therefore giving positive PPD test results. One of the major aims of the research presented in this thesis was to investigate the survival of *M. bovis* BCG in environmental samples, e.g. soil, badger faeces, and water.

The primary method of determining the presence of *M. bovis* BCG in soil would be to use cell extraction and culture methods. There are problems associated with the isolation of *Mycobacterium* species from environmental samples, as it can be both laborious and time consuming (Palomino and Portaels, 1998). Due to the diverse nature of the genus in terms of singular species' growth rates (ranging from a few days to several months) any extraction and culture procedure must be carried out for at least 8 weeks, in order to obtain the majority of the species present in a sample (Williams-Bouyer *et al.*, 2000). This raises an immediate problem of contamination, as agar plates can quickly be overgrown with other indigenous bacteria and fungi present in the sample. Steps need to be taken to ensure the retardation of growth of these organisms, and several methods have been developed, usually involving a decontamination step, involving 1-5 % NaOH addition followed by optional steps of, for example, H_2SO_4 (Iivanainen, 1995), oxalic acid (Iivanainen *et al.*, 1997), or quarternary ammonium compound addition (Dundee *et al.*, 2001). Due to the nature of their cell wall, it is thought that *Mycobacterium* spp are highly resistant to these chemicals, as the thick mycolic acid and lipid outer layer provides a barrier for uptake of the decontaminants (Brennan 2003). This method was initially employed to culture *Mycobacterium* spp. from clinical samples i.e. sputum, lung washes and tissue biopsies, where the level of non-mycobacterial species is much lower than in a soil sample (Oliver *et al.*, 2001). Decontamination of soil samples usually requires the addition of the highest concentrations of the chemicals (Donoghue *et al.*, 1997) and little is known about the effects these decontamination steps have on the *Mycobacterium* species present in a soil sample. There has currently been no

comprehensive survey on the effects of the decontamination of species, both in terms of effects on total cell numbers, and effects on individual species.

A study was done to determine survival rates of *M.bovis* BCG in soil microcosms; using viable cell counts as a measure of cell numbers. It was hypothesised that due to the nature of the selective isolation method, viable counts would be difficult to carry out and not give an accurate representation of this survival and a second method would be needed. This method using molecular techniques was developed to monitor the presence of *M. bovis* BCG marker genes in soil, and quantitation used to estimate cell numbers.

3.2. Aims

The aims of this research were as follows

To determine the effect of a decontamination extraction method on the levels of *M. bovis* BCG inoculated into soil, in order to assess the feasibility of using this method as an accurate measure of the levels of *M. bovis* BCG present in a particular sample.

To create an alternative method of determining the presence of *M. bovis* BCG in soil, negating the use of cell extraction and culture methods. The use of DNA and RNA extraction followed by analysis would be investigated as a way of significantly reducing the time needed to positively identify *M. bovis* BCG in soil.

To monitor survival of *M. bovis* BCG in soil microcosms over time, under a variety of environmental conditions.

To monitor turnover rates of *M. bovis* BCG DNA in soil. This was to determine if the presence of a positive DNA signal from *M. bovis* BCG indicates the presence of viable cells, or if DNA can survive outside of the cell environment in soil for significant periods of time.

3.3 Effect of soil decontamination on *M. bovis* BCG

A typical decontamination method (refer to **Section 2.5**) was chosen for the isolation of *M. bovis* BCG from soil microcosm experiments. The effect on cell numbers of this method was monitored by inoculating 1 g Warwick sterile soil microcosms with 5×10^7 cells. Inoculated soils were incubated at 28°C for 1 h, followed by the decontamination method and plating out cell extracts on to Middlebrook 7H10 agar plates (refer to **Section 2.2**). Plates were incubated at 37°C for 8 weeks. As a control, cells were also extracted using one-quarter strength Ringer's solution, shaken for 10 min, and no further chemical additions. As can be seen in Fig. 3.1, using a simple buffered extraction, cell numbers drop by one log. Whereas no growth was observed using the decontamination method, indicating either a complete killing of the cells, or severely reducing viability of these organisms below the levels of detection using plate count methods.

The effect of the decontamination step on two fast-growing species, *M. smegmatis* and *M. phlei*, was also investigated. Plates were incubated for only two weeks due to the much faster growth rates of these organisms. As shown in Fig. 3.2 there is no complete loss in cell numbers for these two species, however there is still a significant drop in cell numbers of approximately 3-logs. These results pose problems for the extraction and enumeration of *Mycobacterium* species from soil, in particular *M. bovis* BCG. One possible method may be to take into account this loss of cell numbers during extraction, compensating the numbers obtained with the individual cell loss rate for each species. This would only work however with known species where the level of reduction could be calculated prior to extraction. Also this would not take into account the nature of the cells in the soil, would dormant cells be more resistant to the decontamination, and would damaged cells be more susceptible? It is possible that if cell numbers of species are lower than 1×10^3 per gram of soil, they may not be detected at all using this method. Also the use of this method in monitoring *M. bovis* BCG is not practical, as complete cell removal was observed. It was clear from these results that a new method was needed for detecting and enumerating *Mycobacterium* species in soil and other environmental samples, which have a high load of indigenous bacteria.

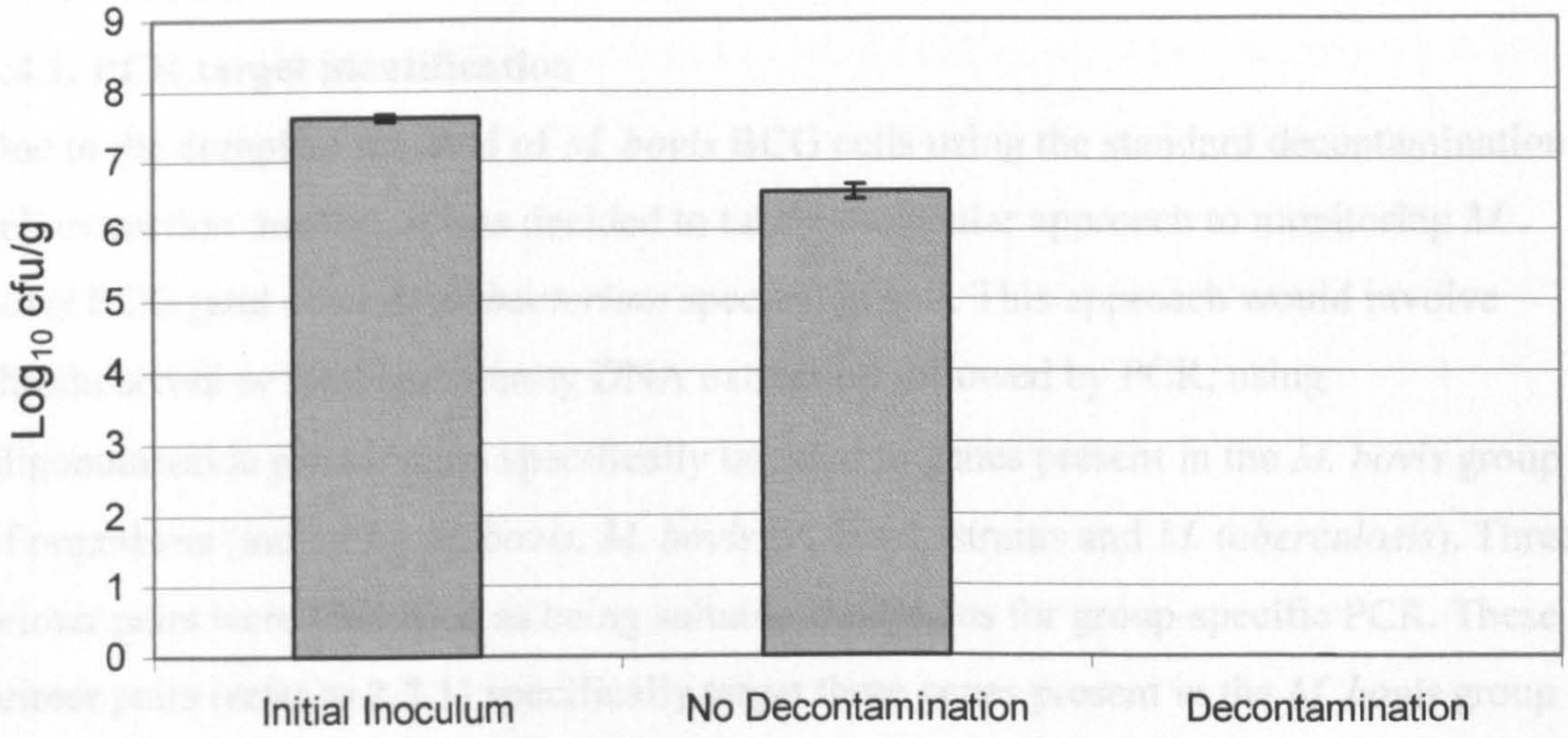


Fig. 3.1. The effect of decontamination of soil microcosms on *M. bovis* BCG culturable cell numbers. Error bars indicate standard deviation of three replicates

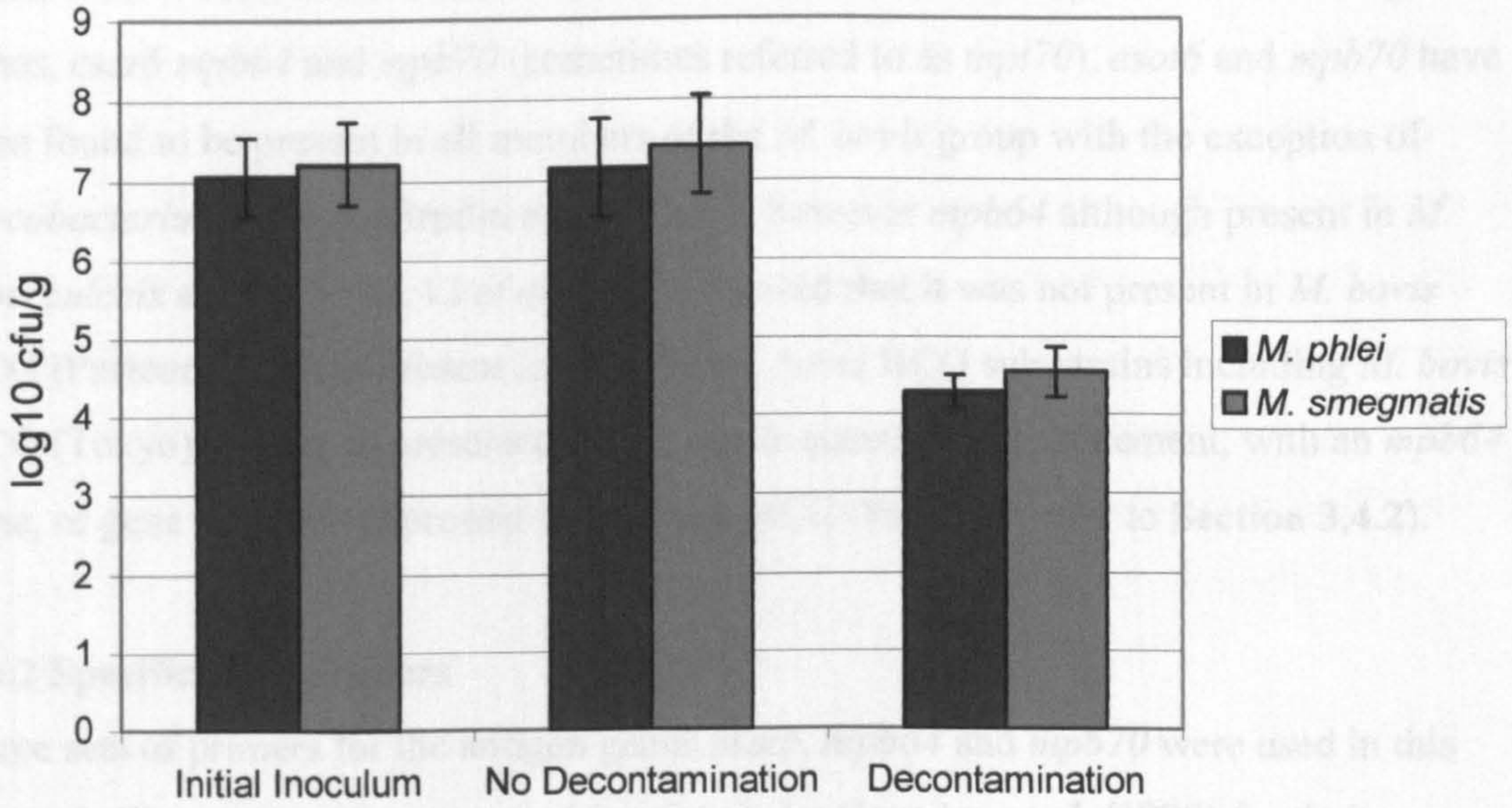


Fig. 3.2. The effect of decontamination of soil microcosms on *M. smegmatis* and *M. phlei* cell numbers. Error bars indicate standard deviation of three replicates

3.4 Detection of *M. bovis* BCG DNA

3.4.1. PCR target identification

Due to the complete removal of *M. bovis* BCG cells using the standard decontamination cell extraction method, it was decided to take a molecular approach to monitoring *M. bovis* BCG (and other *Mycobacterium* species) in soil. This approach would involve chromosomal or total community DNA extraction followed by PCR, using oligonucleotide primer pairs specifically targeted to genes present in the *M. bovis* group of organisms (including *M. bovis*, *M. bovis* BCG sub-strains and *M. tuberculosis*). Three primer pairs were identified as being suitable candidates for group-specific PCR. These primer pairs (refer to **2.7.1**) specifically target three genes present in the *M. bovis* group which encode three proteins that are secreted from cells during active growth and during infection of host animal cells. Once secreted in the host they initiate a strong antigenic immune response and as such the proteins are named antigen proteins, and the genes encoding for them, antigen genes (Alito *et al.*, 1999). Although a variety of these antigen proteins have been discovered, it was decided to focus on three particular encoding genes, *esat6 mpb64* and *mpb70* (sometimes referred to as *mpt70*). *esat6* and *mpb70* have been found to be present in all members of the *M. bovis* group with the exception of *Mycobacterium microti (*Brodin *et al.*, 2002) , however *mpb64* although present in *M. tuberculosis* and *M. bovis*, Li *et al.* (1993) showed that it was not present in *M. bovis* BCG (Pasteur), but was present in all other *M. bovis* BCG sub-strains including *M. bovis* BCG (Tokyo). Evidence presented in this thesis questions this statement, with an *mpb64* gene, or gene homologue present in *M. bovis* BCG (Pasteur) (refer to **Section 3.4.2**).

3.4.2 Specificity of primers

Three sets of primers for the antigen genes *esat6*, *mpb64* and *mpb70* were used in this research. These primers were tested previously by Gormley *et al.* (1999) for their specificity in detecting *M. bovis* group strains, although there was no prior use of the primers in detecting antigen gene presence in soil total community DNA. These primers were used in a PCR on DNA extracted from selected bacterial species listed in table **2.3.1.** The results of this experiment can be seen in Fig. 3.3. Of all the species tested only *M. tuberculosis, M. bovis* and *M. bovis* BCG (Pasteur) gave PCR products for all three

antigen gene primers. It was surprising that *M. bovis* BCG (Pasteur) gave a product for *mpb64*, as, as stated previously the gene should not be present. The culture of *M. bovis* BCG was extracted directly from a vaccine stock, and there can be no doubt as to the authenticity of the strain. To find out whether the primer pair was detecting *mpb64* or priming randomly, the product was cloned and sequenced and this sequence (along with the primer sequences) was imported into The Sanger Institute's, *M. bovis/M. bovis* BCG (Pasteur) Blast server (http://www.sanger.ac.uk/Projects/M_bovis/blast_server.shtml). This program allows pair-wise matching of DNA sequences to sequences present in the *M. bovis* chromosome database (completed) and the *M. bovis* BCG (Pasteur) chromosome database (98.09% coverage). The DNA sequence from BCG relating to *mpb64* matched to a sequence of the correct size in both the *M. bovis* and *M. bovis* BCG (Pasteur) assemblies, indicating the presence of an *mpb64*-like gene in *M. bovis* BCG (Pasteur), indeed Harboe *et al.* (1986) provided evidence for low level production of MPB64 protein in the organism. Secondly, the primer sequences matched up to the same sequence at the appropriate positions, to give a sequence of the same length as the PCR product. Interestingly the published sequence for *mpb64* from *M. bovis* BCG (Tokyo) (Yamaguchi *et al.*, 1989) matches to the *M. bovis* assembly at only 55 % identity and the *M. bovis* BCG (Pasteur) assembly at 60 % identity. This suggests that there is a degree of diversity amongst *mpb64* genes in sub strains of *M. bovis*. In summary the *mbp64* gene primers detected a product of the correct size in *M. bovis* BCG (Pasteur) and not in any other bacterial species tested, and so they were used further in experiments to monitor survival of *M. bovis* BCG in soil. There were no problems associated with *mpb70* and *esat6* as the gene sequences targeted are present in all sub strains of *M. bovis* BCG including Pasteur (Matsuo *et al.*, 1995; Gey Van Pittius *et al.*, 2001).

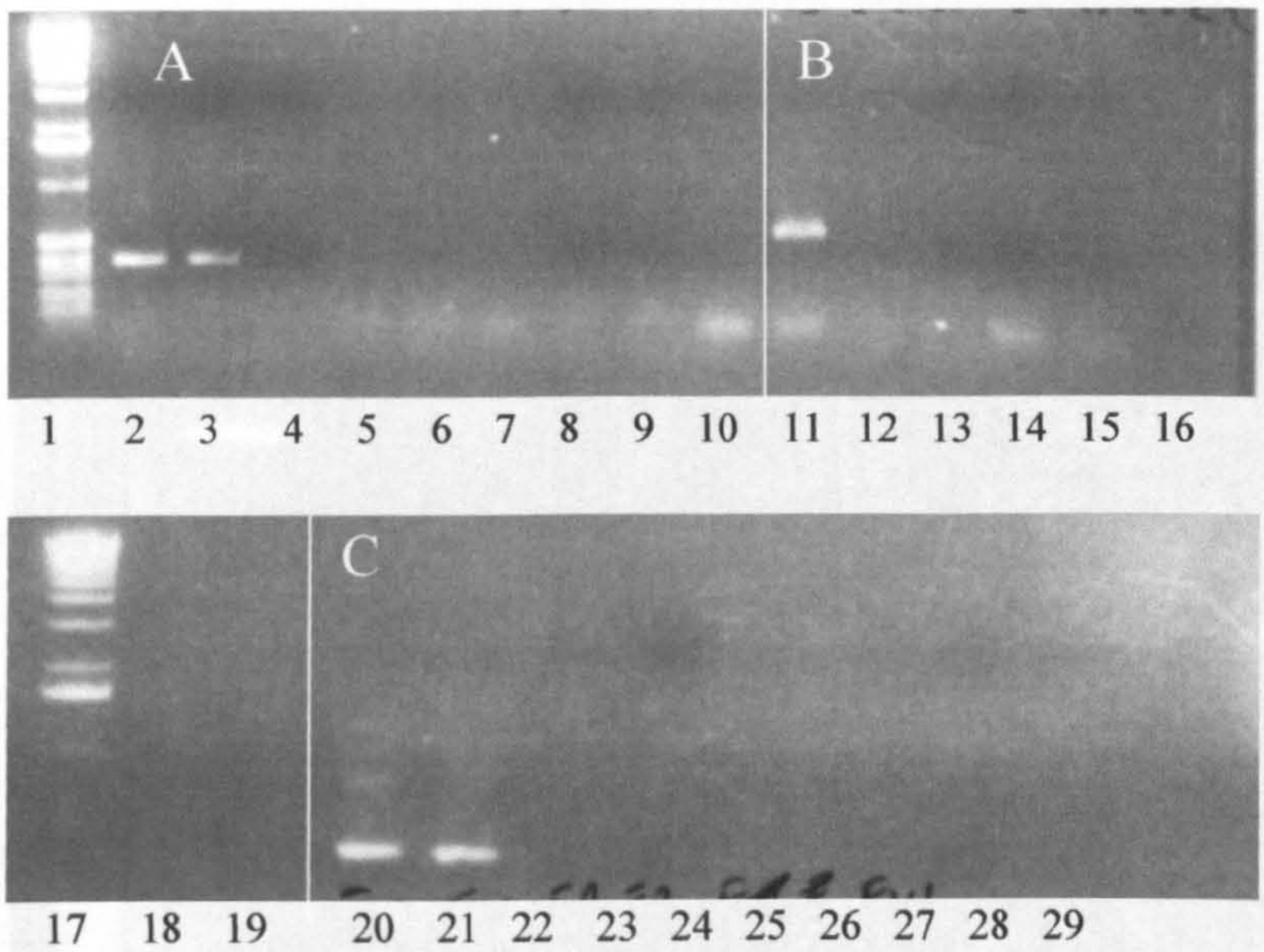


Fig. 3.3 Presence or absence of two antigen genes in different bacterial species (1 % agarose gel of products obtained in PCR), **A**) *mpb64*, lane order is 1) Molecular Markers, 2) *M. bovis*, 3) *Mycobacterium tuberculosis*, 4) *Mycobacterium gordonae*, 5) *Mycobacterium terrae*, 6) *Mycobacterium chitae*, 7) *Mycobacterium gilvum*, 8) *Nocardia brevicatena*, 9) *Rhodococcus coprophilus*, 10) *Streptomyces coelicolor,* **B**) *mpb*70, lane order is 11) *M. bovis*, 12) *Mycobacterium gilvum*, 13) *Mycobacterium fortuitum*, 14) *Mycobacterium gordonae*, 15) *Mycobacterium terrae*, 16) *Mycobacterium chitae*, 17) Molecular Markers, 18) *Nocardia brevicatena*, 19) *Rhodococcus coprophilus*, 20) *Streptomyces coelicolor* and **C**) *esat6,* lane order is 21) *M. bovis*, 22) *Mycobacterium tuberculosis*, 23) *Mycobacterium gordonae*, 24) *Mycobacterium terrae*, 25) *Mycobacterium chitae*, 26) *Mycobacterium gilvum*, 27) *Nocardia brevicatena*, 28) *Rhodococcus coprophilus*, 29) *Streptomyces coelicolor.*

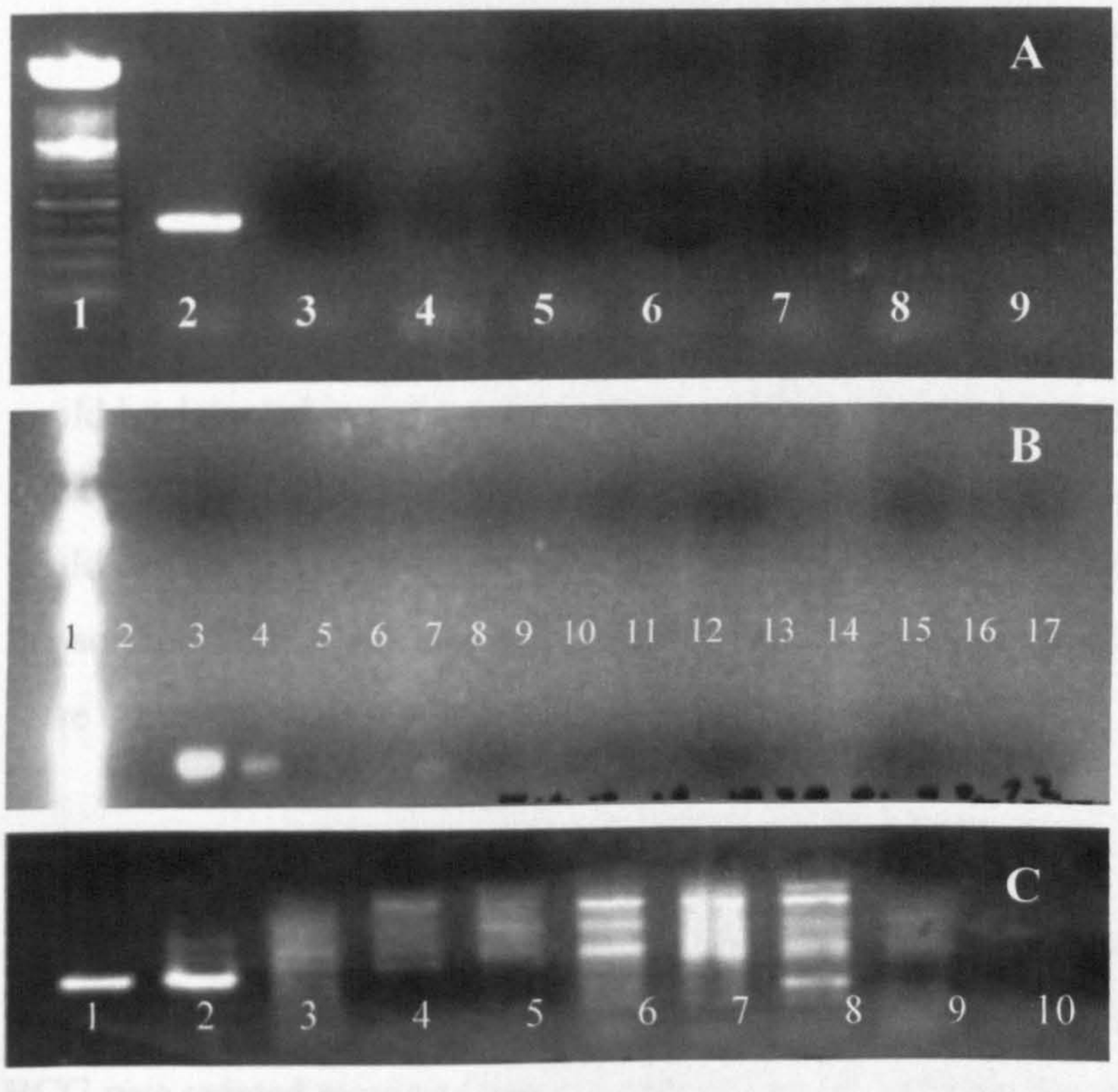


Fig. 3.4 Background level of three antigen genes in soils from different geographical locations (1 % agarose gel of products obtained in PCR), **A)** *mpb64*, lane order is 1) Molecular Markers, 2) *M. bovis* BCG, 3) Greece soil, 4) Italy soil, 5) Cuba soil, 6) Cotswolds soil, 7) Warwick soil 8) North Wales soil, 9) Negative Control. **B)** *mpb70*, lane order is 1) Molecular Markers, 2) Negative control, 3) *M. bovis* BCG, 4) *M. bovis* 5,6) Greece soil, 7,8) Italy soil, 9,10) Cuba soil,11,12) Cotswolds soil, 13,14) Warwick soil 15,16,17) North Wales soil, and **C)** *esat6* lane order is 1) *M. bovis*, 2) *M. bovis* BCG, 3) Greece soil, 4) Italy soil, 5) Cuba soil, 6) Cotswolds soil, 7,8) Warwick soil 9) North Wales soil, 10) Negative Control.

To test for background levels of the target genes in soil, total community DNA was extracted from the soils given in **Section 2.4.** PCR was carried out on these DNA samples and no PCR products were detected in any of the soils from Greece, Italy, Cuba, and the Cotswolds, Warwick and North Wales (UK) (Fig. 3.4). This indicates that the two target antigen genes *mpb64* and *mpb70* do not commonly occur in the environment. However, despite the specificity in detecting *esat6* in only *M. bovis* group species (although some non-specific banding is noted when targeting *Str. coelicolor* DNA), no specific product could be detected in soil total community DNA. The predominant bands found in this PCR were excised, cloned and sequenced, but no significant homology to other genes could be found. This indicates that there are regions of DNA in some bacterial species in the soil that can be detected by this primer set. Because of this only *mpb64* and *mpb70* were used in further work.

3.4.3 Limits of detection

It was important, therefore, to establish limits of detection for the two gene targets, both from chromosomal DNA and from total community DNA. A dilution series of *M. bovis* BCG was created ranging from 10^9 cells/ml to 10^1 cells/ml for chromosomal DNA, each of these dilutions was inoculated into separate 1g soil microcosms in volumes of 1 ml. DNA was then extracted from all microcosms and all dilutions, and PCR carried out using the two antigen gene primer sets (all as in **Section 2.8.2**). Results can be seen in Fig. 3.5. Limits of detection for both primer sets were 10^1 cells/ml for chromosomal DNA, and 10^2 cells/g soil for total community DNA. Due to this low limit of detection it is important to carry out PCR on the total volume of DNA extracted rather than just one aliquot, in order to make sure a sample is truly negative, as the gene could be missed if a single aliquot is used.

Fig. 3.5 PCR detection limits of **A)** *mpb64* and **B)** *mpb70* using chromosomal DNA. DNA was extracted from a dilution series of *M. bovis* BCG Lanes are as follows **A)** 1) 10^1 cells/ml, 2) 10^2 cells/ml. 3) 10^3 cells/ml, 4) 10^4 cells/ml, 5) 10^5 cells/ml, 6) 10^6 cells/ml, 7) 10^7 cells/ml, 8) 10^8 cells/ml and **B)** 1) 10^8 cells/ml, 2) 10^7 cells/ml, 3) 10^6 cells/ml, 4) 10^5 cells/ml, 5) 10^4 cells/ml, 6) 10^3 cells/ml, 7) 10^2 cells/ml, 8) 10^1 cells/ml.

3.4.4 Quantitation of PCR

The use of standard PCR can only distinguish between the presence or absence of a gene target in a particular sample, and cannot provide an accurate quantitation of the amount of gene target present. There are currently two main methods for use in quantitative PCR, TaqMan (Heid *et al.* 1996) and LightCycler techniques (Wittwer *et al.*, 1997; Mölling *et al.*, 2002). Both methods can be used in two forms. Firstly measuring product accumulation in the PCR through the amount of fluorescence of a probe bound to double stranded DNA (Kuhne and Oschmann, 2002) and secondly, by measuring the amount of SYBR-Green intercalated into double stranded DNA throughout the PCR (Bach *et al.* 2002), or measuring the amount of fluorescent-labelled probe throughout the PCR Both these methods have problems with accurate reporting of gene numbers from soil total community DNA, mainly in the form of fluorescence quenching by auto-fluorescence of humic acid contamination in the DNA preparation. Humic acids are known to co-purify with DNA during extraction procedures, and currently some DNA extractions may have to be diluted to overcome this. This dilution may be of concern if the number of target genes in the sample is at, or slightly higher than the limit of detection of the PCR. Any dilution applied would mean the target gene numbers would be below the limit of detection and therefore would not be successfully amplified in a PCR. Also, the effectiveness of fluorescence quantitation depends on the DNA extraction method employed and also varies on the species being detected. In work done by Filion *et al.* (2002) using LightCycler/SYBR-Green quantitation, limits of detection ranged from 10^1 cells/ g soil of the arbuscular mycorrhizal fungus *Glomus intraradices*, to 10^3 cells/g soil for the plant pathogen *Fusarium solani* f. sp. *phaseoli.* Pilot experiments were carried out using mpb64 primers in a SYBR-Green quantitative PCR using the LightCycler, however despite repeated attempts no accurate results could be obtained. The *M. bovis* BCG soil dilution series was used, and little correlation between dilution and fluorescence output was monitored Fig. 3.6 gives a melting curve graph indicating the huge amount of non-specific fluorescence present in the experiments.

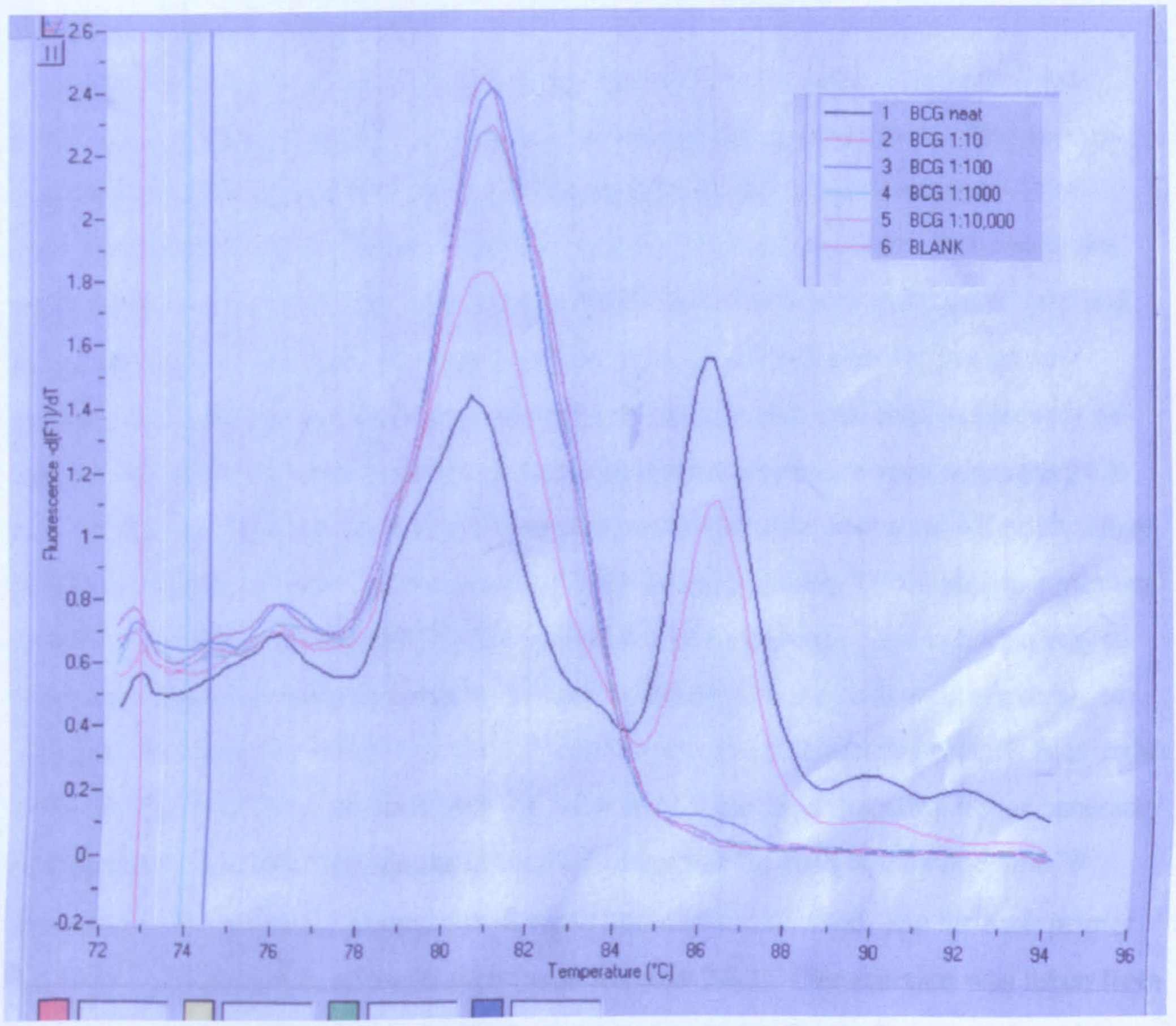


Fig 3.6. Melting curve of a dilution series of *M. bovis* BCG obtained using real time fluorescent PCR targeted to *mpb64*. Neat represents 10^8 cells/g soil.

3.4.5 Direct gel quantitation

A method was then established for the quantitation of PCR products through direct analysis of gel images negating the use of fluorescent-based quantitation. This process uses the TotalLab suite of programs (in this case the 1d gel analysis program) from Phoretix Products (UK). TotalLab analyses gel images and can accurately identify the pixel intensity of a particular band, with normalisation controls to standardise gels and allow for cross-gel comparison. One problem in using this program (or indeed any analysis software) on gels from normal PCR reactions, is that quantitation can only be carried out on PCR products resulting from reactions that were stopped when the PCR was still linear. PCR reactions proceed in an exponential state with a cut off point where the accumulation of more double stranded DNA product ceases. This is due to a number of reasons, namely, all free dNTPs being used and primers being depleted. One way to overcome this is to make sure that both primers and dNTPs are both well in excess, or secondly to determine where this cut off point occurs and to quantify products before this point. It was decided to proceed with the latter in this case and therefore it was necessary to discover where this "lag" phase in the PCR occurred for both *mpb64* and *mpb70* primer sets. To this end 2 separate PCR experiments were devised, one for each primer set, with 35 identical reactions in each (as in **Section 2.8.1**). One reaction was taken from the PCR machine after each cycle. Each reaction was immediately frozen, and products visualised after all cycles were complete. The gels were then analysed for pixel intensity of each band and a curve created (Fig. 3.7 and Fig. 3.8).

As can be seen the PCRs for both primer sets follow the standard exponential curve for a PCR, with a linearity cut-off point occurring at cycles 30 for both sets. It was therefore decided that for both sets of primers, PCRs would be stopped at 28 cycles (where the reaction was in a linear mode) and quantitation of products carried out at this point. This cut-off point can be varied dependent on the number of target genes present in the unknown sample, if the target gene number is low i.e. 10^1 to 10^3 gene copies per g soil, the "lag" phase would occur later in the PCR than if the target number was high i.e. 10^7 to 10^8 gene copies. Pilot experiments need to be carried out to roughly estimate the gene

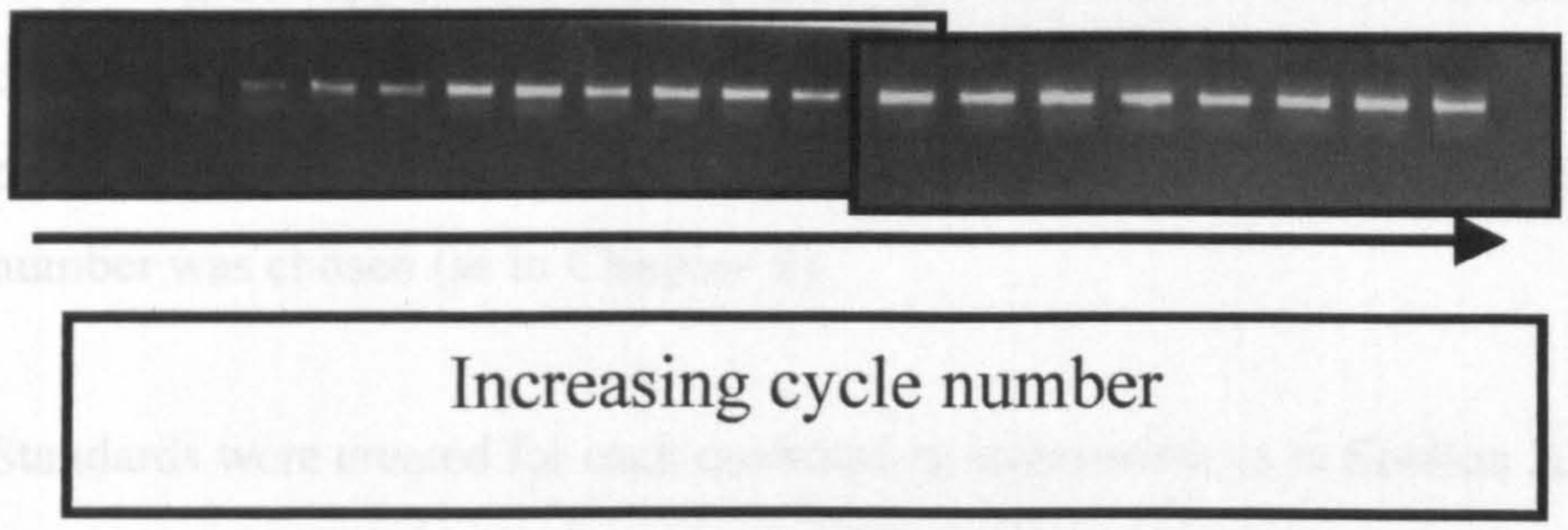
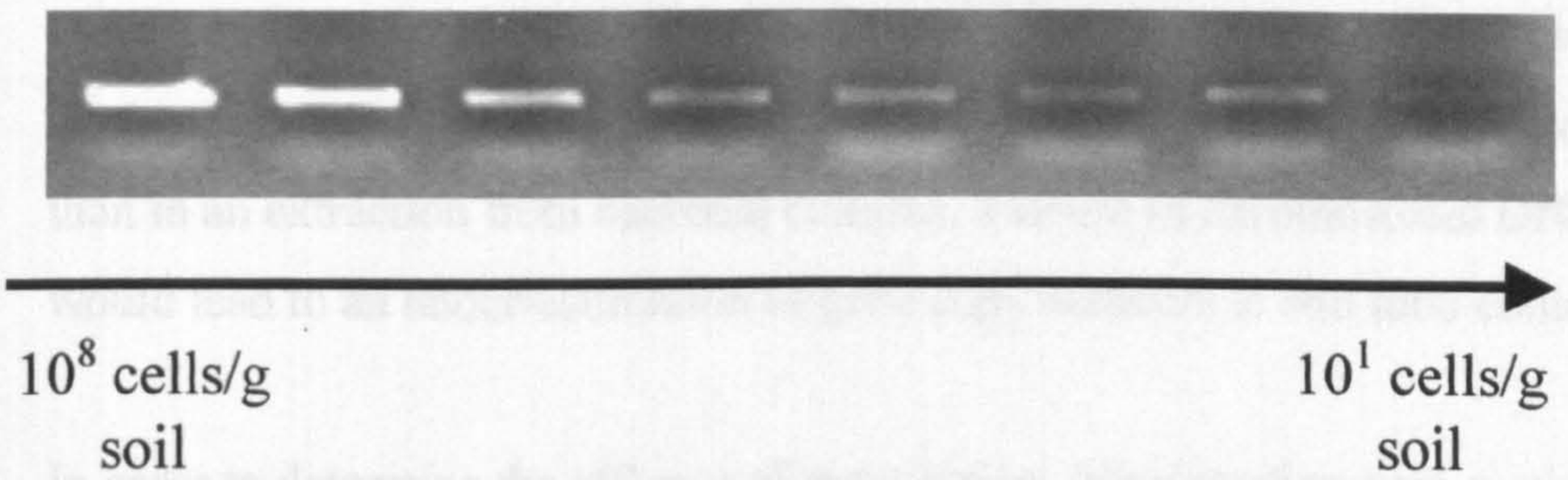


Fig. 3.7 Products derived to determine the exponential phase of a PCR using *mpb70* targeted primers.



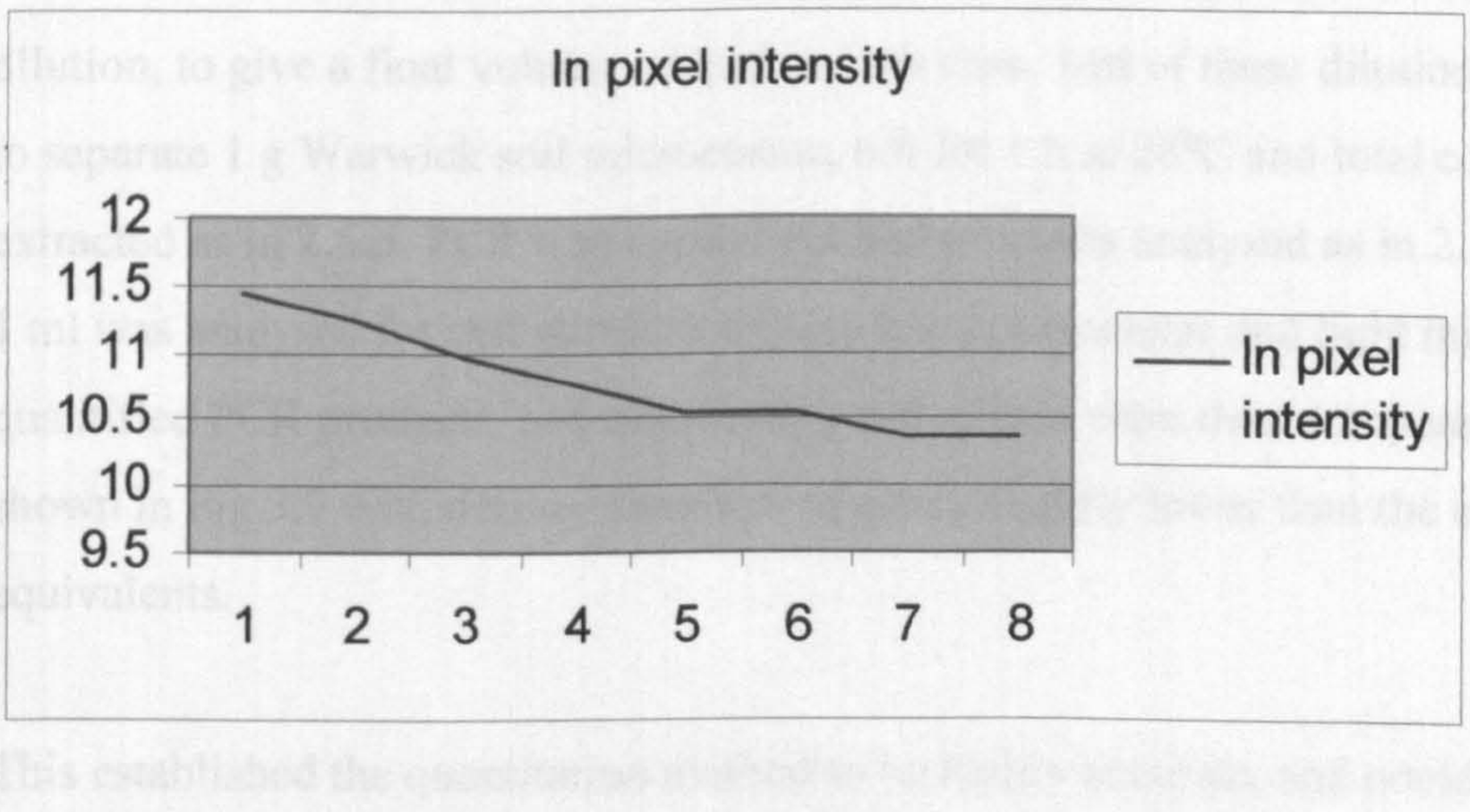


Fig. 3.8. Dilution curve of *mpb70* primer products targeted to *M. bovis* BCG DNA. This creates standards for use in PCR quantitation.

copy numbers. For the survival of *M. bovis* BCG experiments, pilot studies gave the range of gene copy numbers between 10^3 and 10^6, therefore 28 cycle reactions were chosen. For later experiments on the occurrence of *M. bovis* in environmental samples, pilot studies gave target numbers as between 10^2 and 10^3, therefore a higher cycle number was chosen (as in **Chapter 5**).

Standards were created for each quantitation experiment as in **Section 2.8.2.** These standards were used in each separate PCR experiment and were electrophoresed alongside unknowns on each gel. These standards allowed for cross-gel comparison. This also shows the importance of the nature of positive quantitation controls in quantitative PCR, i.e. the controls used for creating a calibration curve should undergo exactly the same extraction protocol as unknowns, in this case inoculation into soil and extraction of total community DNA. Due to the nature of DNA extraction from soil, more DNA is lost than in an extraction from bacterial cultures. The use of chromosomal DNA as standards would lead to an under-estimation of gene copy numbers in soil total community DNA.

In order to determine the efficacy of quantitation, blind studies were carried out. A culture of *M. bovis* BCG was randomly diluted into five aliquots, each of a different dilution, to give a final volume of 2ml in each case. 1ml of these dilutions was then added to separate 1 g Warwick soil microcosms, left for 1 h at 28°C and total community DNA extracted as in **2.6.3**. PCR was carried out and products analysed as in 2.8. The remaining 1 ml was analysed for cell numbers using a haemocytometer and light microscopy. The quantified PCR products, and microscopy cell counts were then compared, results are shown in Fig 3.9 with derived numbers of genes slightly lower than the culturable cell equivalents.

This established the quantitation method to be highly accurate, and could be used successfully to establish gene numbers in further microcosm experiments.

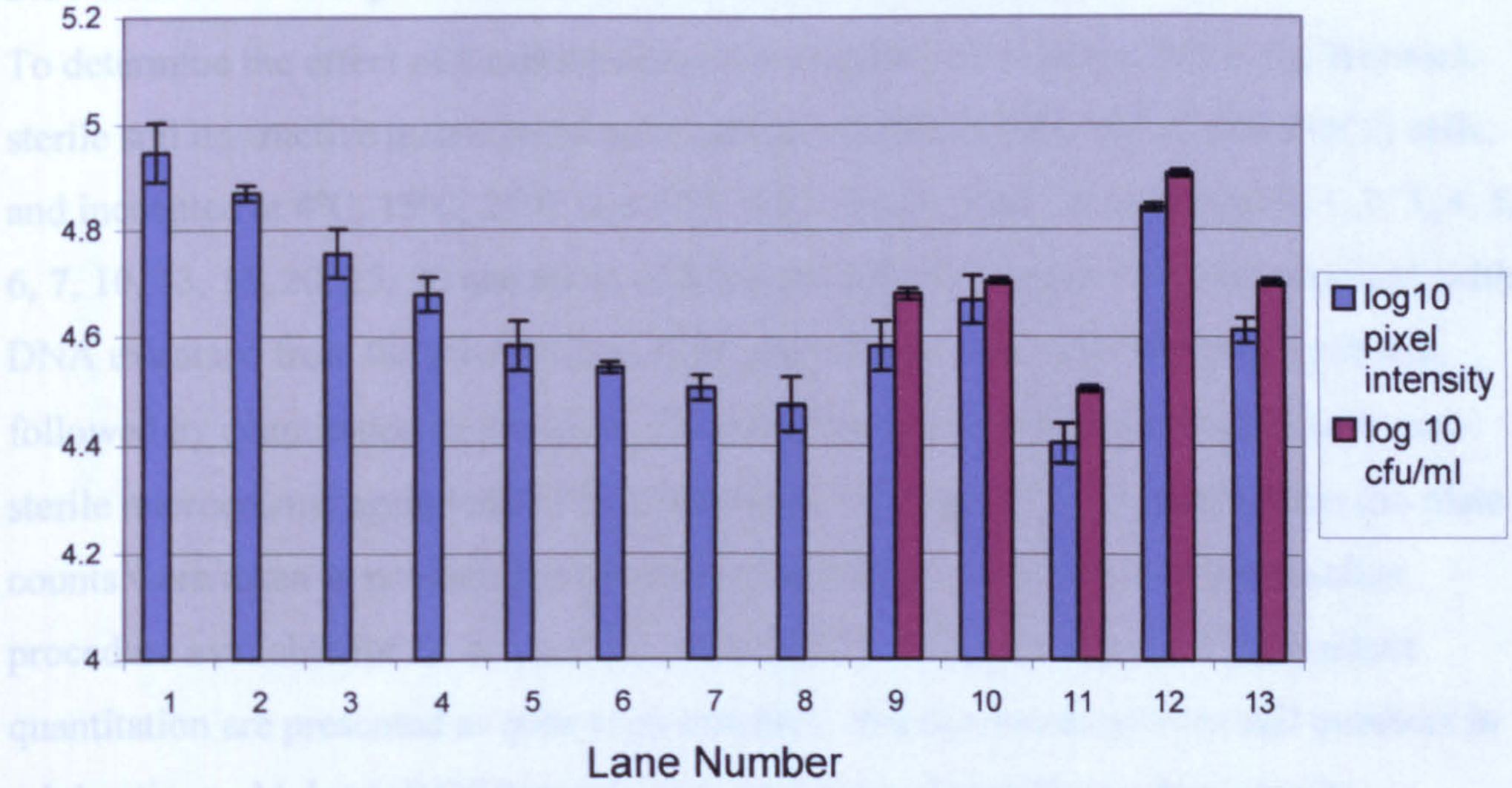


Fig 3.9. Blind testing of quantitative PCR, 1) 10^1 cells/ml, 2) 10^2 cells/ml. 3) 10^3 cells/ml, 4) 10^4 cells/ml, 5) 10^5 cells/ml, 6) 10^6 cells/ml, 7) 10^7 cells/ml, 8) 10^8 cells/ml, 9) dilution 1, 10) dilution 2, 11) dilution 3, 12) dilution 4, 13) dilution 5.

3.5. Effect of temperature on *M. bovis* BCG survival

To determine the effect of temperature on the longevity of *M. bovis* BCG, 1 g Warwick sterile soil destructive microcosms were set up, inoculated with 10^6 *M. bovis* BCG cells, and incubated at 4°C, 15°C, 25°C and 37°C. Cell counts were taken at days 0, 1, 2, 3, 4, 5, 6, 7, 10, 13, 16, 20, 25, 30 and 60 as in **2.5.2** and **2.9**. This experiment was repeated, with DNA extracted from the microcosms, PCR carried out using *mpb70* targeted primers, followed by quantitation of products. The experiment was repeated a final time in non-sterile microcosms, again with DNA extractions, PCR and product quantitation (no plate counts were taken in non-sterile soil experiments due to no accurate cell extraction procedure available for *M. bovis* BCG, refer to **3.3**) Although data for PCR product quantitation are presented as gene copy numbers, this equates directly to cell numbers in a 1:1 ratio, as *M. bovis* BCG has only one gene copy of *mpb70* per chromosome.

3.5.1 Sterile soil microcosms – culturable cell counts

Results of plate count data for sterile soil are shown in Fig. 3.10. After an initial count of 10^6 cfu/g soil a decrease is seen at all temperatures. The counts at 4 °C decrease most rapidly and after day 16, there are no culturable *M. bovis* BCG cells present. At 15°C, counts decrease gradually from 10^6 cfu/g soil at day 0 to 6×10^3 cfu/g soil by day 10. Between days 10 and 13 there is a 1 log-fold drop and by day 20 there were no culturable cells present. The decrease in counts at 25°C is less marked between days 0 and 10 dropping from 10^6 cfu/g soil to 2×10^4 cfu/g soil. Counts continue to decrease and by day 60 less than 10 cfu/g soil were present. At 37°C the decrease is the lowest for all four temperatures, with a drop from 10^6 cfu/g soil to 5×10^5 cfu/g soil between days 0 and 10. This comparatively slow decrease rate continues from day 10, although by day 60 less than 10 cfu/g soil are present. No culturable *M. bovis* BCG cells could be detected after day 60 in microcosms incubated at all four temperatures. Decline rates in $\log_{10}$ decrease/day were calculated for the four temperatures and were 4.59, 4.35, 4.11 and 4.07 for 4°C, 15°C, 25°C and 37°C respectively (Fig 3.11).

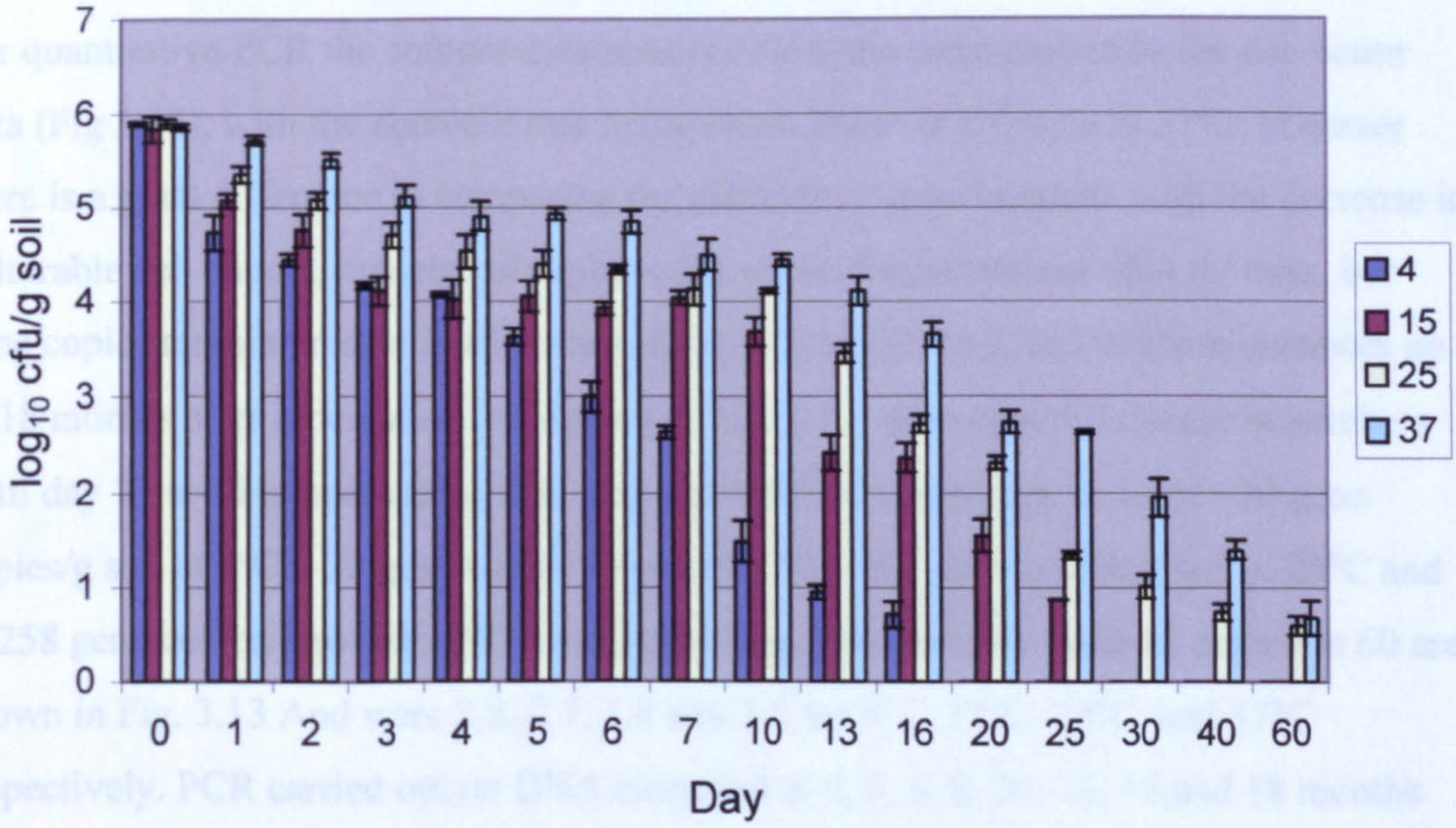


Fig. 3.10 Survival of *M. bovis* BCG in 1 g sterile soil microcosms at four different temperatures (°C), monitored by culturable cell counts.

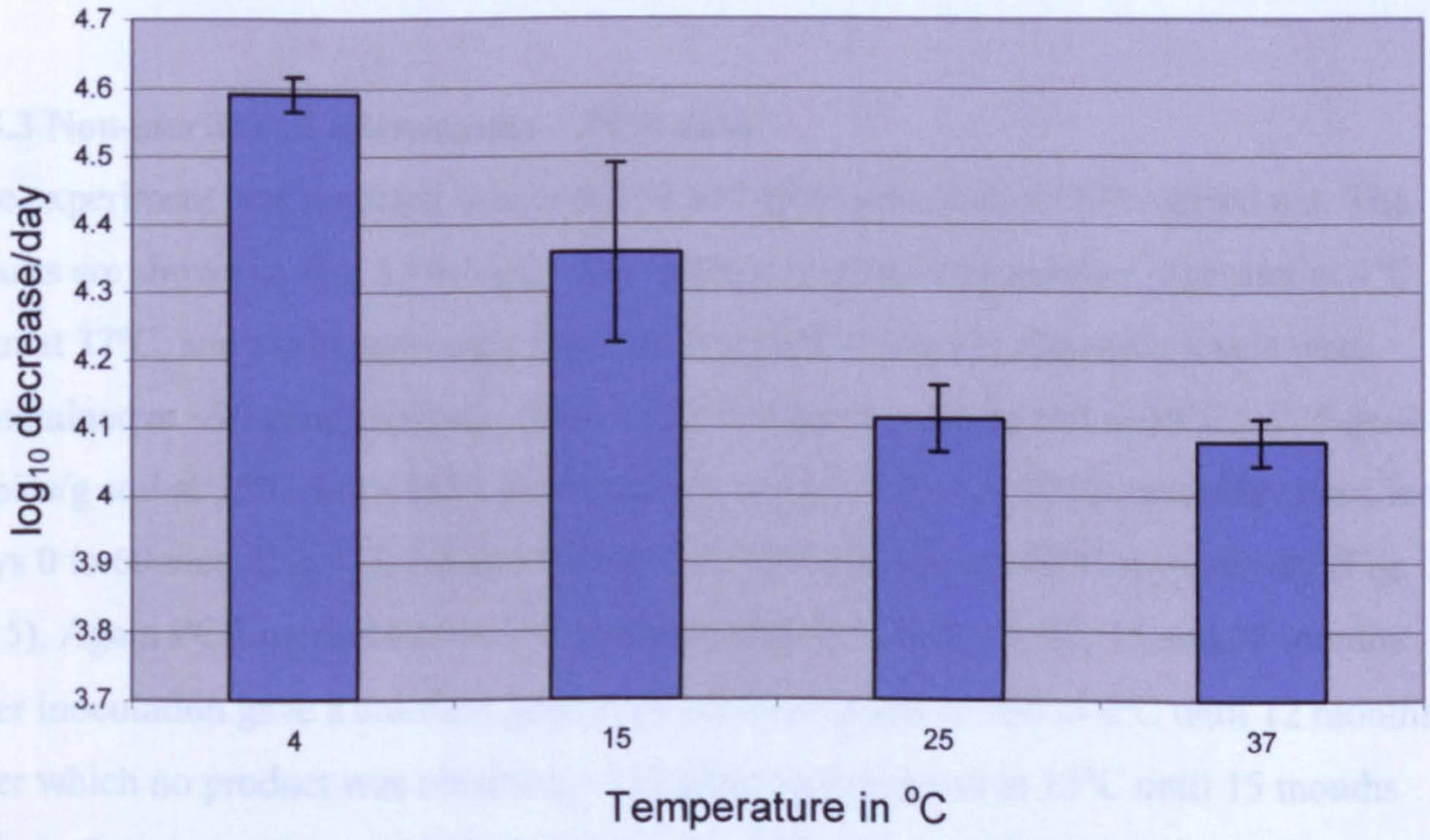


Fig. 3.11. Decrease/day of cfu/g soil of *M. bovis* BCG over time in 1 g sterile soil microcosms at four different temperatures (°C).

3.5.2 Sterile soil microcosms – PCR data

For quantitative PCR the comparative results follow the same pattern as the cell count data (Fig 3.12), with the decrease rate being much lower at 4°C than at 37°C, however there is a great difference in comparing the decrease in gene numbers with the decrease in culturable cell counts, namely culturable cells are no longer present after 60 days, but gene copies remain present in microcosms for beyond 60 days, and in some instances up to 18 months after inoculation. As shown in Fig. 3.12, gene copies decrease in numbers until day 13 at all temperatures, then begin to level off reaching a value of ~20 gene copies/g soil at 4°C, ~30 gene copies/g soil at 15°C, ~630 gene copies/g soil at 25°C and ~1258 gene copies/g soil at 37°C. Log_{10} gene copy decrease/day rates for days 0 to 60 are shown in Fig. 3.13 And were 2.8, 2.7, 1.8 and 1.5 for 4°C, 15°C, 25°C, and 37°C respectively. PCR carried out on DNA extracted at 4, 5, 6, 8, 10, 12, 15,and 18 months after inoculation gave a constant gene copy number/ g soil of ~20 at 4°C until 10 months after which no product was obtained, ~25 gene copies/ g soil at 15°C until 12 months where after no product could be detected, and ~500 gene copies/g soil and ~900 gene copies/g soil for 25°C and 37°C respectively. No product was obtained at 25°C and 37°C at 18 months.

3.5.3 Non-sterile soil microcosms – PCR data

The experiment was repeated in non-sterile soil with quantitative PCR carried out. The results are shown in Fig. 3.14 Again the decrease in gene copy number is greater at 4°C than at 37°C, and again gene copy numbers level off at day 13. Constant levels were maintained at ~90 gene copies/g soil at 4°C, ~218 gene copies/g soil at 15°C, ~275 gene copies/g soil at 25°C and ~1659 gene copies/g soil at 37°C. Log10 decrease/day rates for days 0 to 60 were 2.2, 1.7, 1.5 and 0.8 for 4°C, 15°C, 25°C, and 37°C respectively (Fig. 3.15). Again PCR carried out on DNA extracted at 4, 5, 6, 8, 10, 12, 15,and 18 months after inoculation gave a constant gene copy number/ g soil of ~50 at 4°C until 12 months after which no product was obtained, ~150 gene copies/ g soil at 15°C until 15 months where after no product could be detected, and ~200 gene copies/g soil and ~1250 gene copies/g soil for 25°C and 37°C respectively.

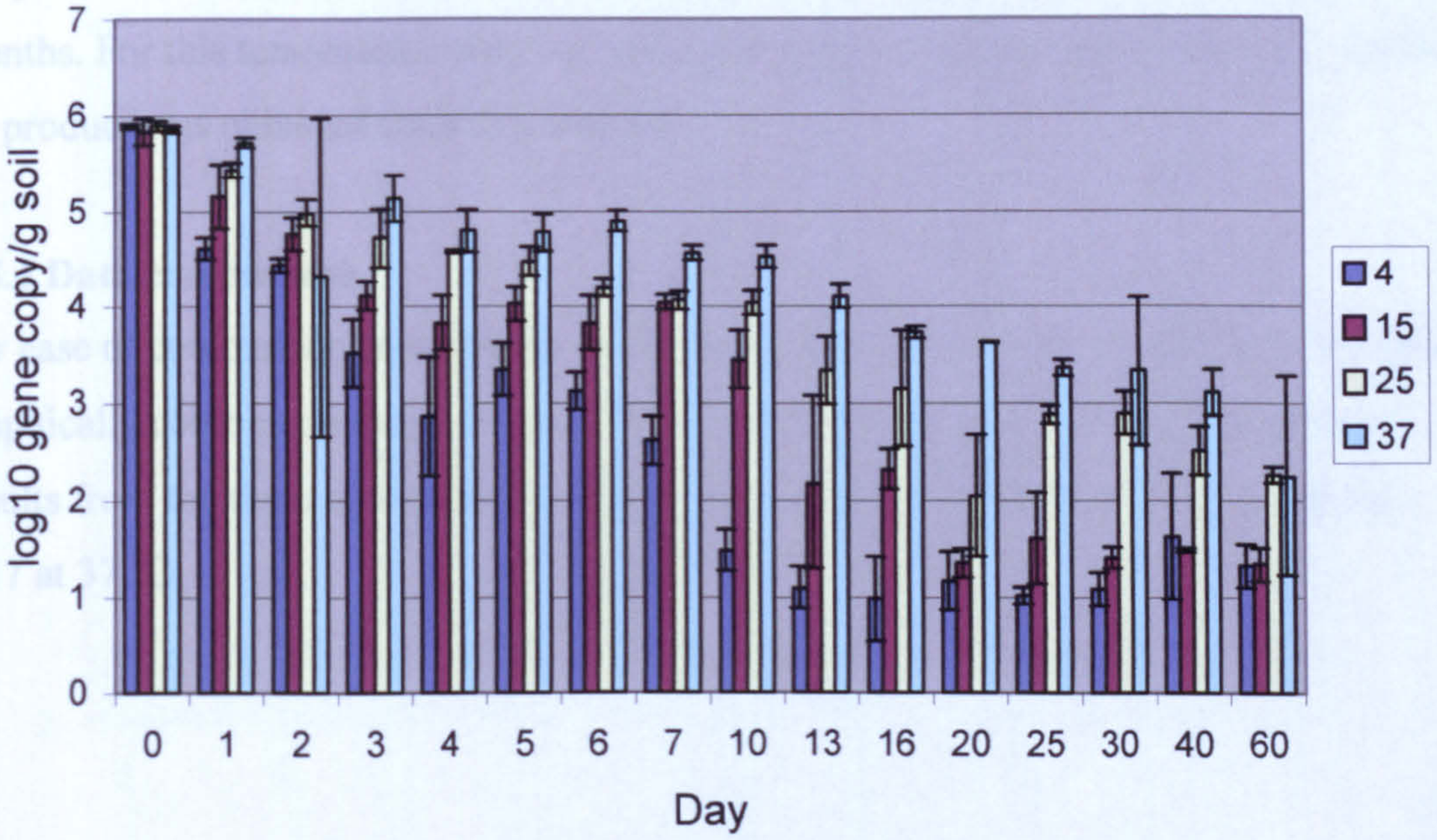


Fig. 3.12 Survival of *M. bovis* BCG in 1 g non-sterile soil microcosms at four different temperatures (°C), monitored by direct gel quantitation of PCR products.

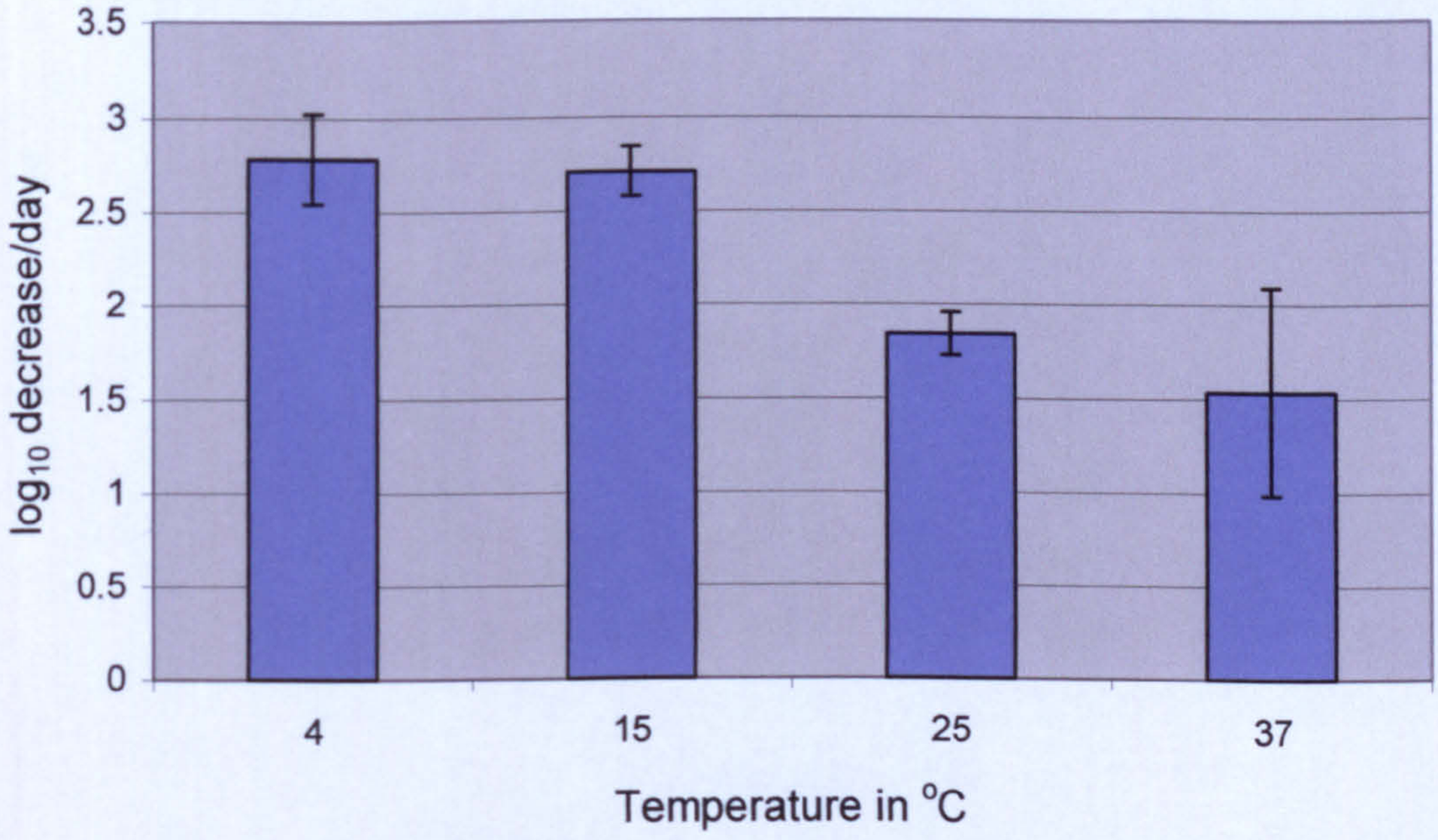


Fig. 3.13. Decrease/day of gene copies/g soil of *mpb70* from M. *bovis* BCG in non-sterile soil microcosms at four different temperatures (°C).

No product was obtained at 25 °C at 15 months, at 37 °C product was still obtained at 18 months. For this temperature only one further time-point sample was taken at 22 months, no product was obtained from this sample.

3.5.4 Data comparison

For ease of comparison results from the three separate microcosm experiments have been graphically combined using 4 °C and 37 °C as an example. Fig. 3.16 gives combined results from the three quantitations used of survival of *M. bovis* BCG at 4 °C and Fig. 3.17 at 37 °C.

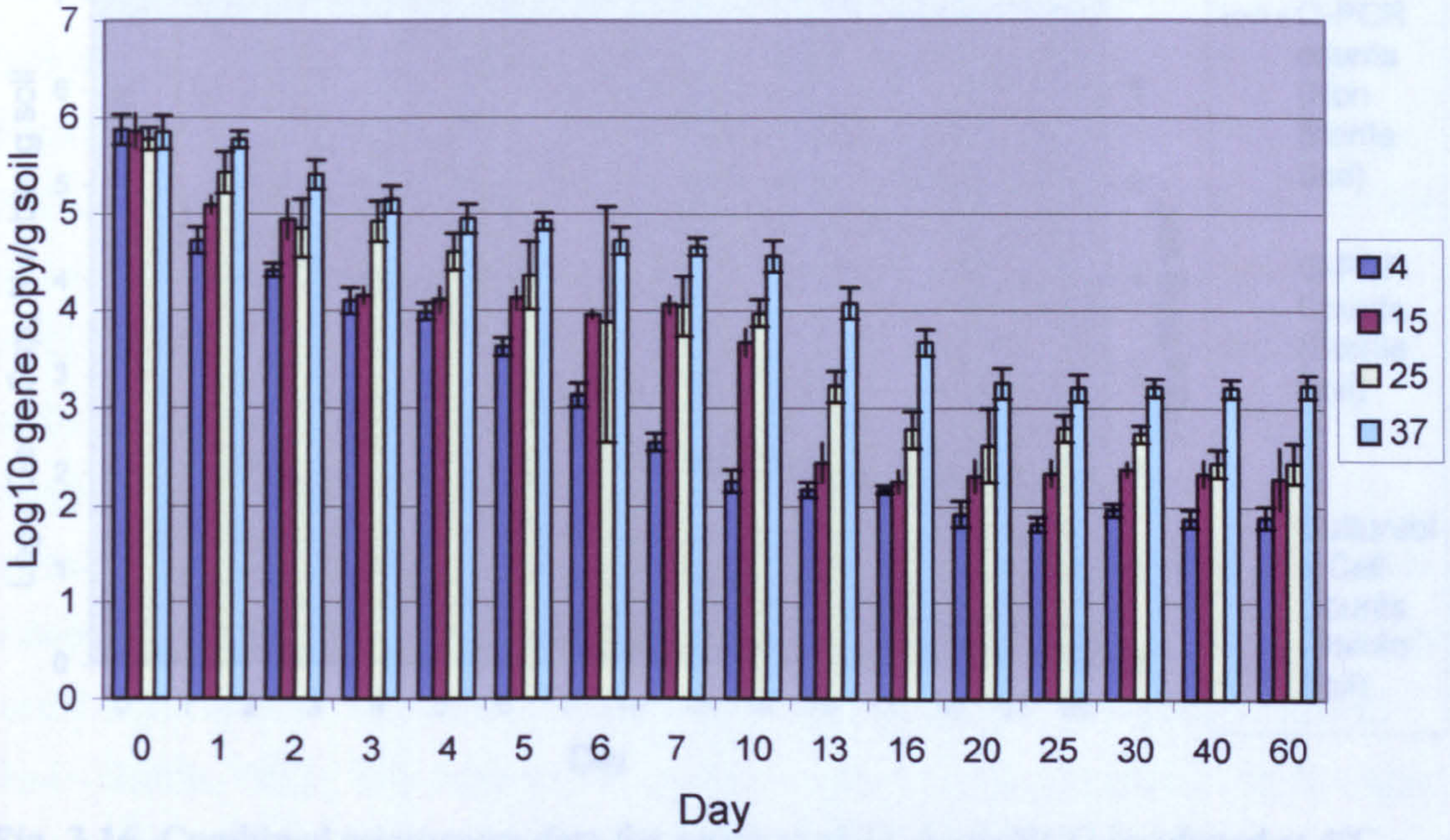


Fig. 3.14. Survival of *M. bovis* BCG in 1 g sterile soil microcosms at four different temperatures, monitored by direct gel quantitation of PCR products.

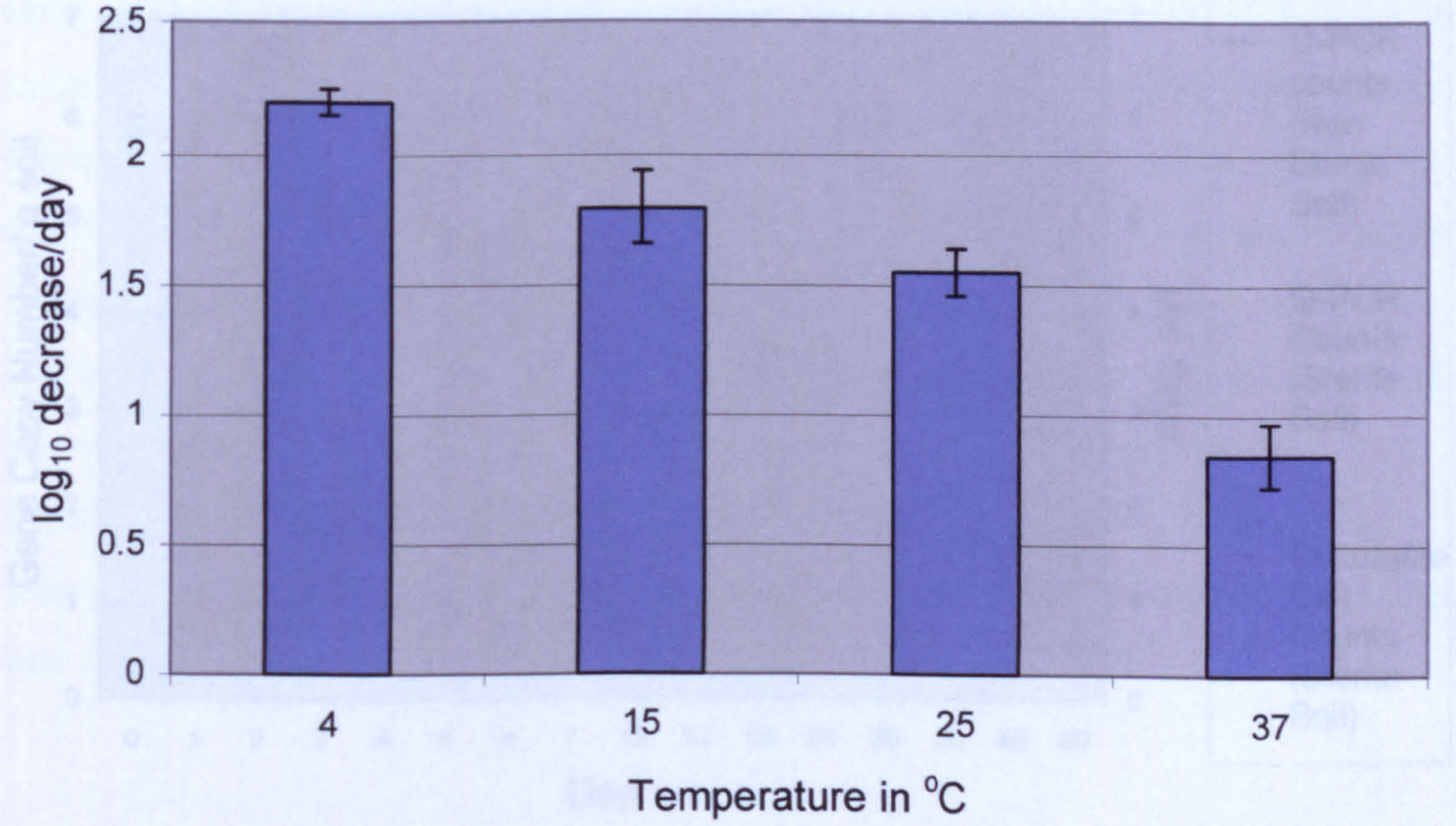


Fig. 3.15. Decrease/day rate of *mpb70* from M. *bovis* BCG in non-sterile soil at four different temperatures, monitored by direct gel quantitation of PCR products.

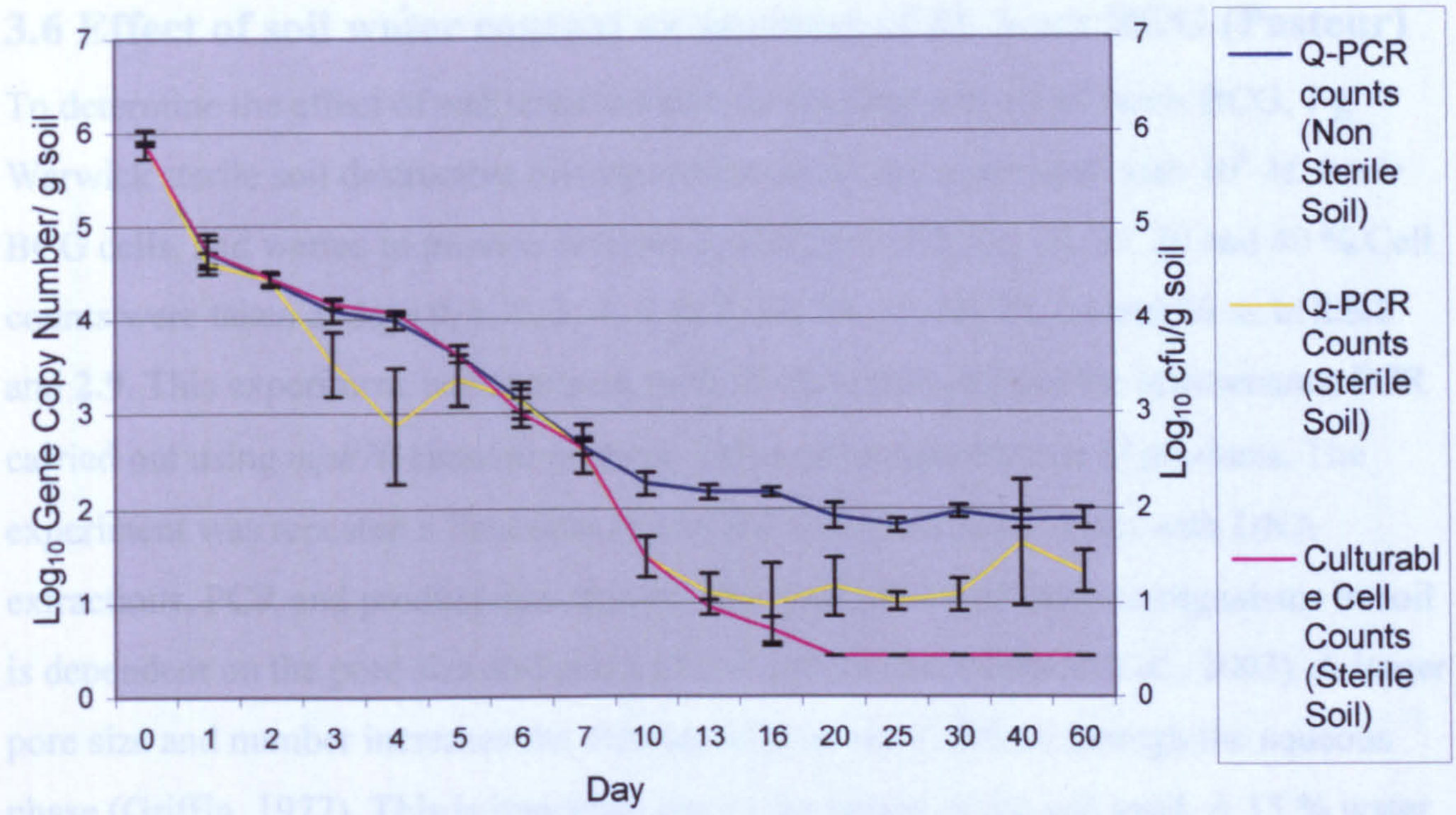


Fig. 3.16. Combined microcosm data for survival of *M. bovis* BCG incubated at 4°C. Three experiments are shown, non-sterile soil gene counts, sterile soil gene counts and sterile soil cell counts.

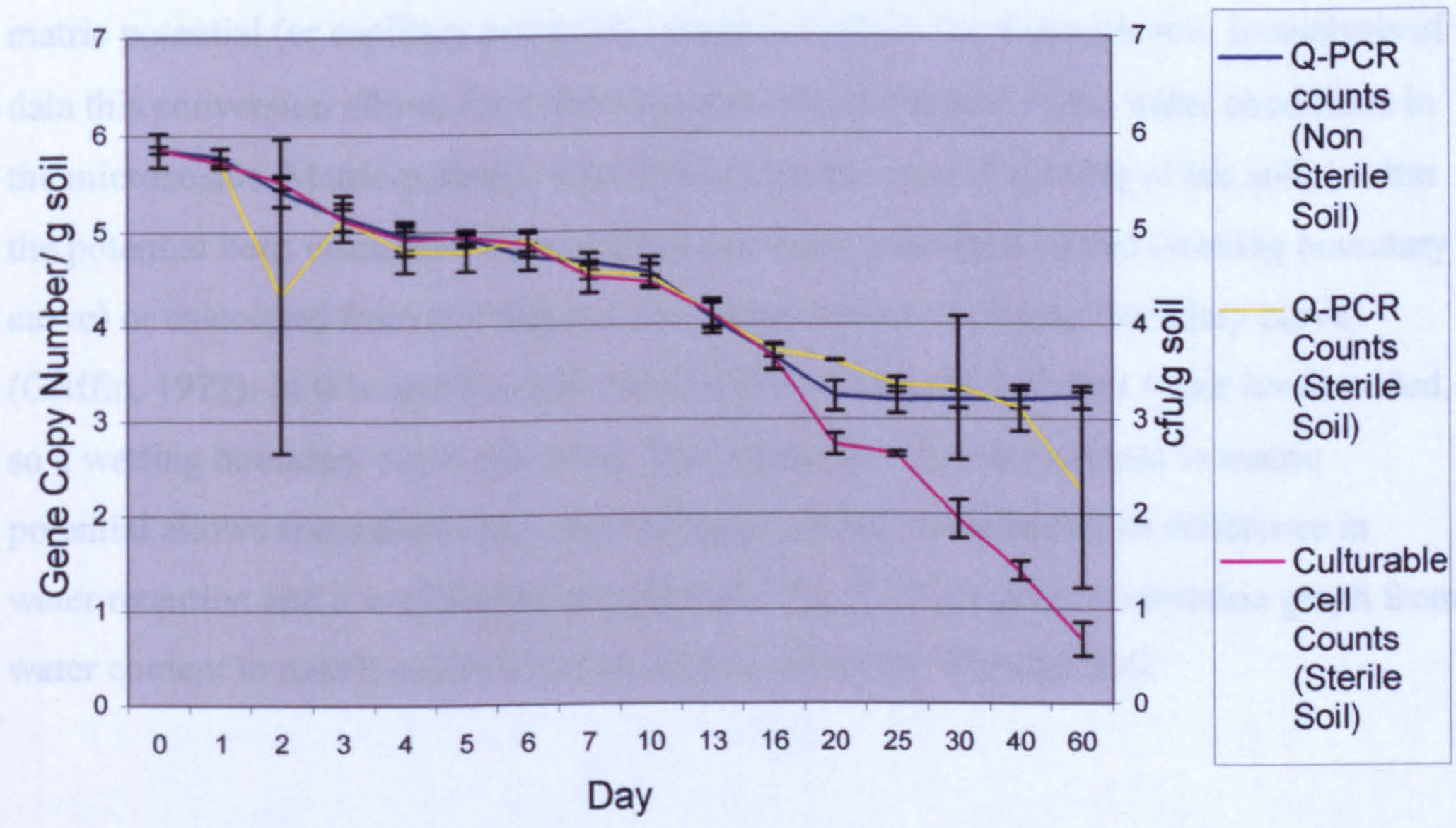


Fig. 3.17. Combined microcosm data for survival of *M. bovis* BCG incubated at 37°C. Three experiments are shown, non-sterile soil gene counts, sterile soil gene counts and sterile soil cell counts.

3.6 Effect of soil water content on survival of *M. bovis* BCG (Pasteur)

To determine the effect of soil water content on the longevity of *M. bovis* BCG, 1 g Warwick sterile soil destructive microcosms were set up, inoculated with 10^6 *M. bovis* BCG cells, and wetted to provide final water contents of 5, 10, 15, 20, 30 and 40 %.Cell counts were taken at days 0, 1, 2, 3, 4, 5, 6, 7, 10, 13, 16, 20, 25, 30 and 60 as in **2.5.2** and **2.9.** This experiment was repeated, with DNA extracted from the microcosms, PCR carried out using *mpb70* targeted primers, followed by quantitation of products. The experiment was repeated a final time in non-sterile microcosms, again with DNA extractions, PCR and product quantitation. The availability of water to organisms in soil is dependent on the pore size and space of soil particulates (Treves *et al.*, 2003). A larger pore size and number increases the distance oxygen must diffuse through the aqueous phase (Griffin, 1972). This is important due to the nature of the soil used. A 15 % water content in Warwick soil (mainly clay based) (Wellington *et al.*, 1990) would be extremely dry in comparison to a 15 % water level in a sandy soil, due to the higher water retention of clay. The water level percentages used in this experiment were converted into matric potential (or capillary potential) values individual for Warwick soil. In analysis of data this conversion allows for a more accurate representation of the water conditions in the microcosms. Matric potential also depends on the state of wetting of the soil, i.e. has the potential been calculated from soil that has been dried then wetted (wetting boundary curve) or calculated from soil that has been dried from wet (drying boundary curve) (Griffin, 1972). In this case the soils were air dried, then the different water levels added, so a wetting boundary curve was used. The conversion of water content to matric potential allows comparison between soil types taking into account the difference in water retention and it availability to organisms. Fig. 3.18 gives the conversion graph from water content to matric potential (expressed in -KPa) for Warwick soil.

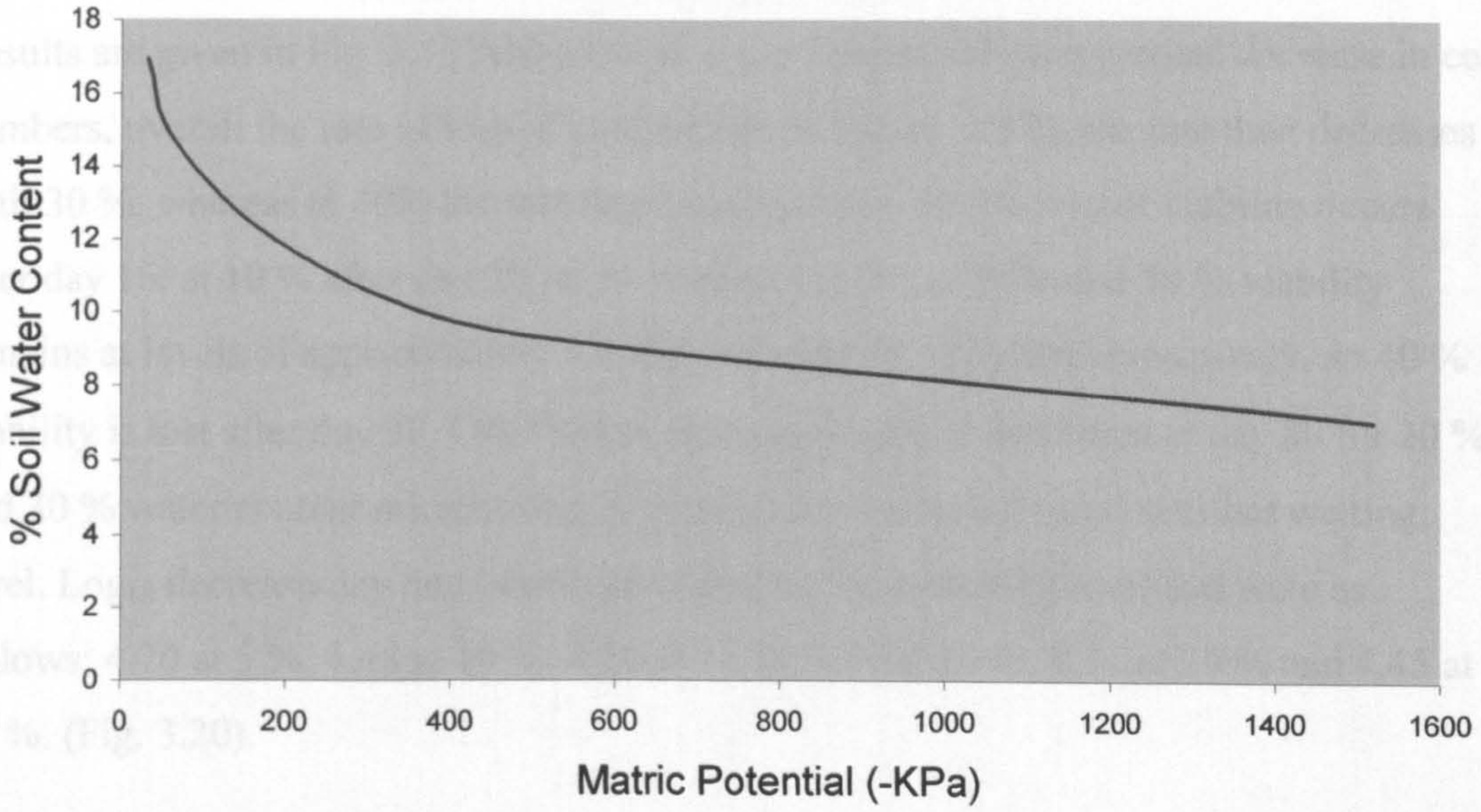


Fig 3.18. Moisture characteristic curve for Warwick soil. x axis denotes minus KPa values.

3.6.1 Sterile soil microcosms – culturable cell counts

Results are given in Fig. 3.19 Although all water contents show a gradual decrease in cell numbers, overall the rate of loss of culturability is higher at 5 %, the rate then decreases until 30 %, whereas at 40% the rate begins to increase. At 5% loss of viability occurs after day 16, at 10 % after day 20, at 15 % after day 20, at 20 % and 30 % viability remains at levels of approximately 10 cfu/g soil and 30 cfu/g soil respectively. At 40 % viability is lost after day 30. One further time point sample was taken at day 80 for 20 % and 30 % water content microcosms, with no viable cells recovered at either wetting level. Log_{10} decrease/day rates were calculated for each wetting level and were as follows: 4.70 at 5 %, 4.48 at 10 %, 4.50 at 15 %, 4.29 at 20 %, 4.11 at 30 % and 4.45 at 40 %. (Fig. 3.20).

3.6.2 Sterile soil microcosms – PCR data

Results are shown in Fig 3.21 Comparison of rates of loss with plate count data, indicates that PCR data follows a similar pattern, with loss decreasing as water content increases, up until 30 %, then rising thereafter. By day 10 5 % water content microcosms gave the greatest loss with gene copies/g soil going from an initial level of 7.5×10^6 to 8×10^1. The rate of loss at other water contents was much lower, at 40 % the gene copies/g soil were 5×10^3 by day 10. Levels at day 10 for 10 % and 15 % were 8×10^2 and 8×10^3 gene copies/g soil respectively. The lowest rate of loss by day 10 was seen in 20 % and 30 % wetting levels, which reached 10^4 and 1×10^4 gene copies/g soil respectively. In this case no total loss of *mpb70* target was observed at any wetting level up to day 60. Further samples were taken at months 4, 5, 6, 8, 10, 12, 15, 18, with total loss of gene copies/g soil occurring at 4 months for 40 %, 6 months for 5 % and 10 % and 16 months for 15 %. Loss of target gene in microcosms did not occur at 18 months for 20 %, and 30 % wetting levels. For these water contents only, one further sample was taken at 22 months, with no product detected at this time point. Decrease/day rates are given in Fig. 3.22.

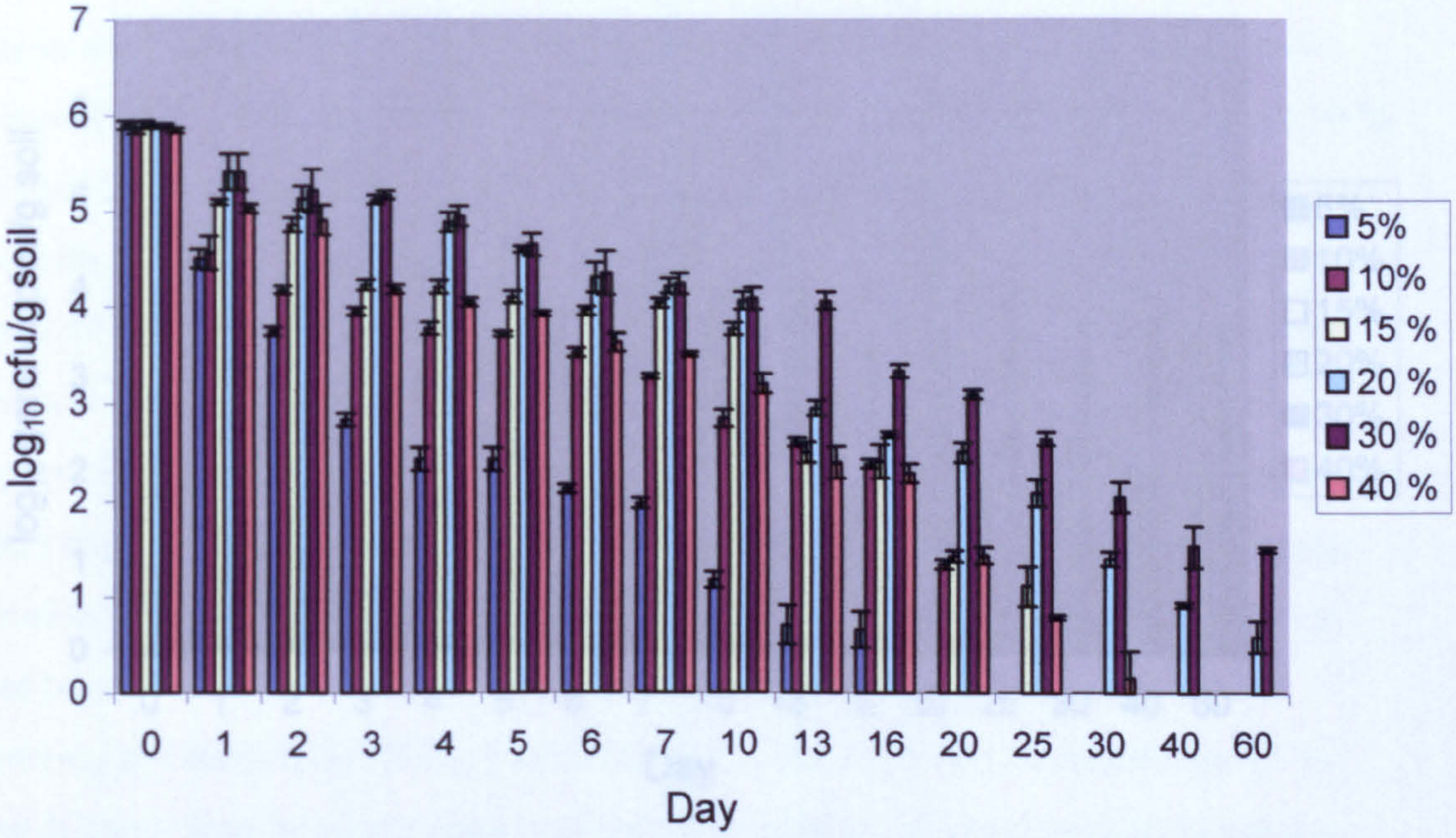


Fig. 3.19 Survival of *M. bovis* BCG over time in sterile soil microcosms, at different soil wetting levels. (culturable cell counts).

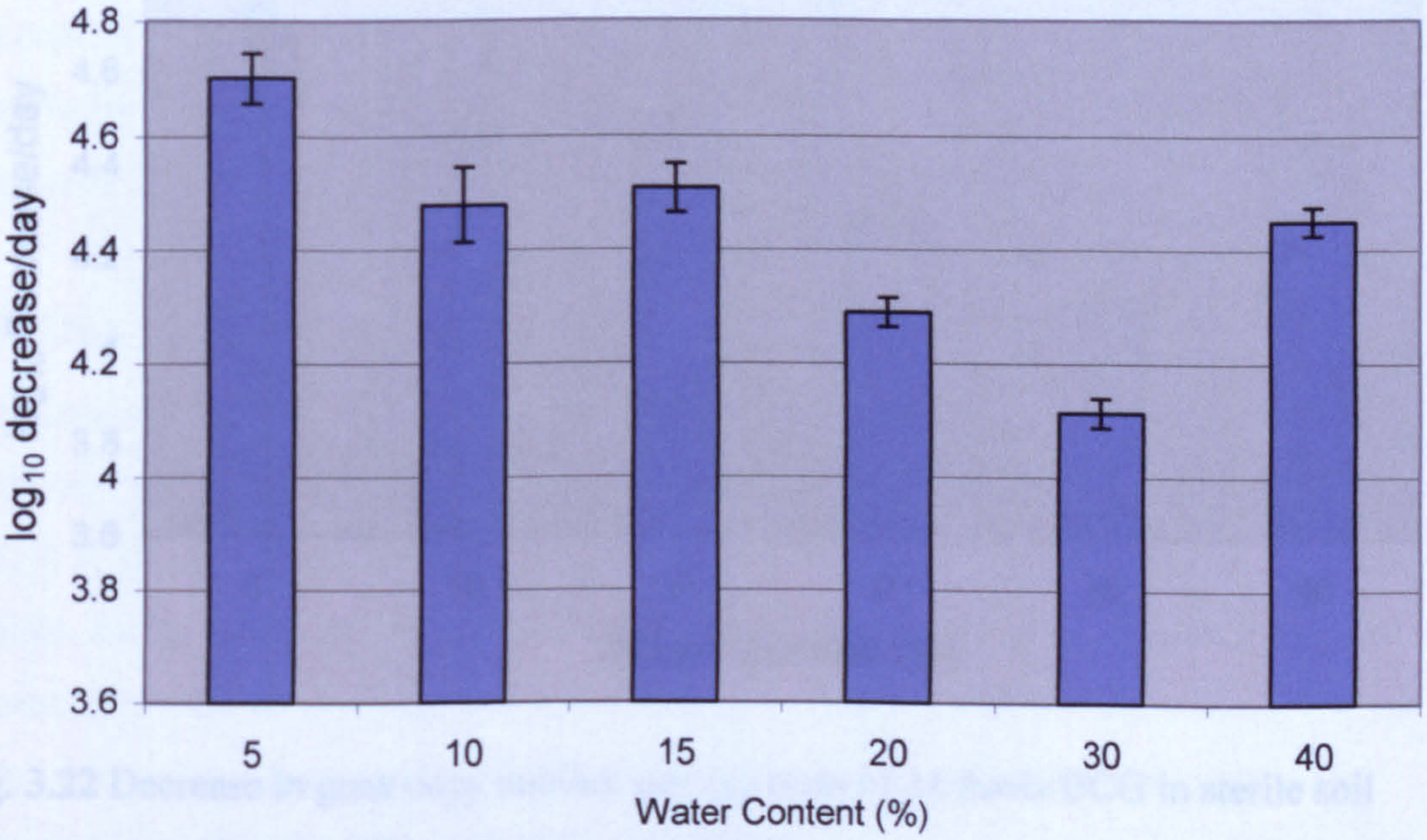


Fig. 3.20 Decrease of cell numbers per day rates of *M. bovis* BCG in sterile soil microcosms held at different soil wetting levels (culturable cell counts).

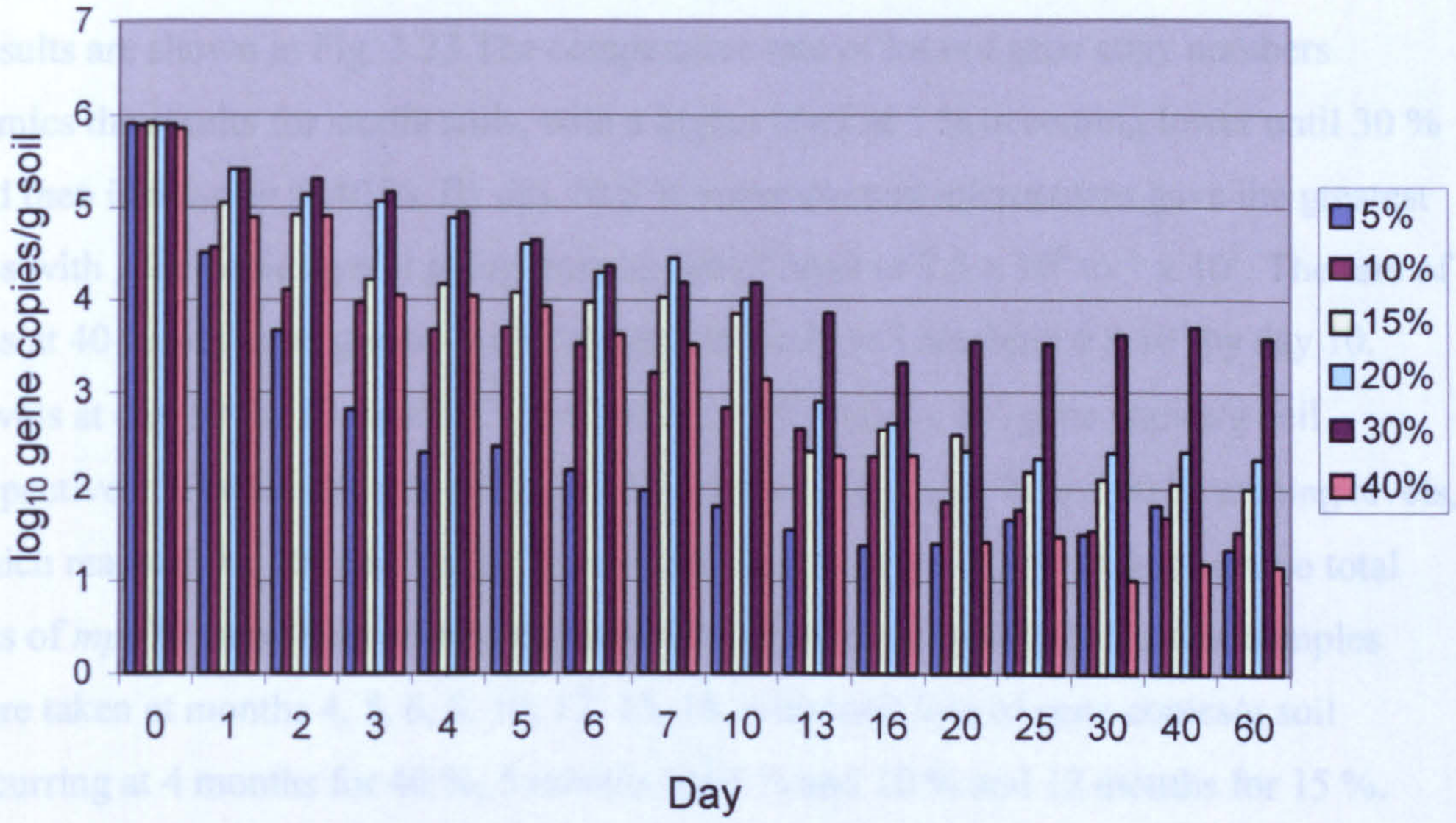


Fig. 3.21 Survival of *M. bovis* BCG in sterile soil microcosms, held under different soil wetting levels (PCR gene count data).

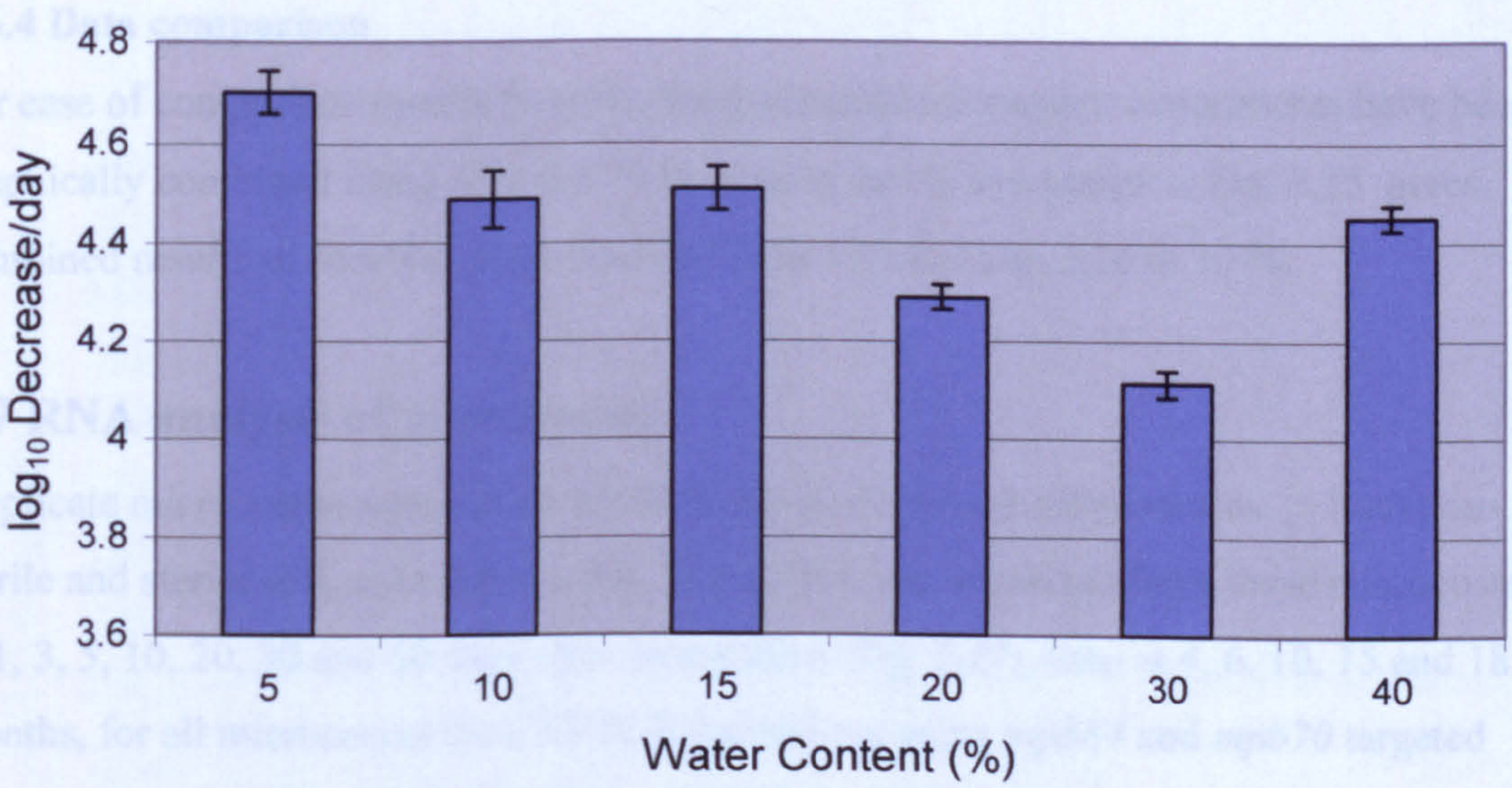


Fig. 3.22 Decrease in gene copy number per day rates of *M. bovis* BCG in sterile soil microcosms held under different soil wetting levels.

3.6.3 Non-sterile soil microcosms – PCR data

Results are shown in Fig. 3.23 The comparative rate of loss of gene copy numbers mimics the results for sterile soils, with a higher level at 5 % becoming lower until 30 % and then increasing at 40 %. By day 10 5 % water content microcosms gave the greatest loss with gene copies/g soil going from an initial level of 7.5×10^6 to 1×10^2. The rate of loss at 40 % was even greater with the gene copies/g soil reaching 6×10^1 by day 10. Levels at day 10 for 10 % and 15 % were 7.5×10^2 and 4×10^3 gene copies/g soil respectively. The lowest rate of loss by day 10 was seen in 20 % and 30 % wetting levels, which reached 9×10^3 and 5×10^4 gene copies/g soil respectively. In this case no total loss of *mpb70* target was observed at any wetting level up to day 60. Further samples were taken at months 4, 5, 6, 8, 10, 12, 15, 18, with total loss of gene copies/g soil occurring at 4 months for 40 %, 5 months for 5 % and 10 % and 12 months for 15 %. Loss of target gene in microcosms occurred at 18 months for 20 %, and 30 % wetting levels. Decrease/day rates are given in Fig. 3.24.

3.6.4 Data comparison

For ease of comparison results from the three separate microcosm experiments have been graphically combined using 5 % and 30 % wetting levels as examples. Fig. 3.25 gives combined results of survival of *M. bovis* BCG at 5 % and Fig. 3.26 at 30 %.

3.7 RNA analysis of microcosms

Duplicate microcosms were set up for all water contents and temperatures, in both non-sterile and sterile soil, as in **3.5** and **3.6**. Total RNA was extracted from these microcosms at 1, 3, 5, 10, 20, 30 and 60 days after inoculation (Fig. 3.27), then at 4, 6, 10, 15 and 18 months, for all microcosms then RT PCR carried out using *mpb64* and *mpb70* targeted primers. Products for both antigen genes were found at day 0 in all microcosms, but not at day 1, or any subsequent time point. RT-PCR was also carried on total community RNA using primers specifically targeted to the 16S rRNA gene of *Mycobacterium* spp and will be described in **Chapter 5.**

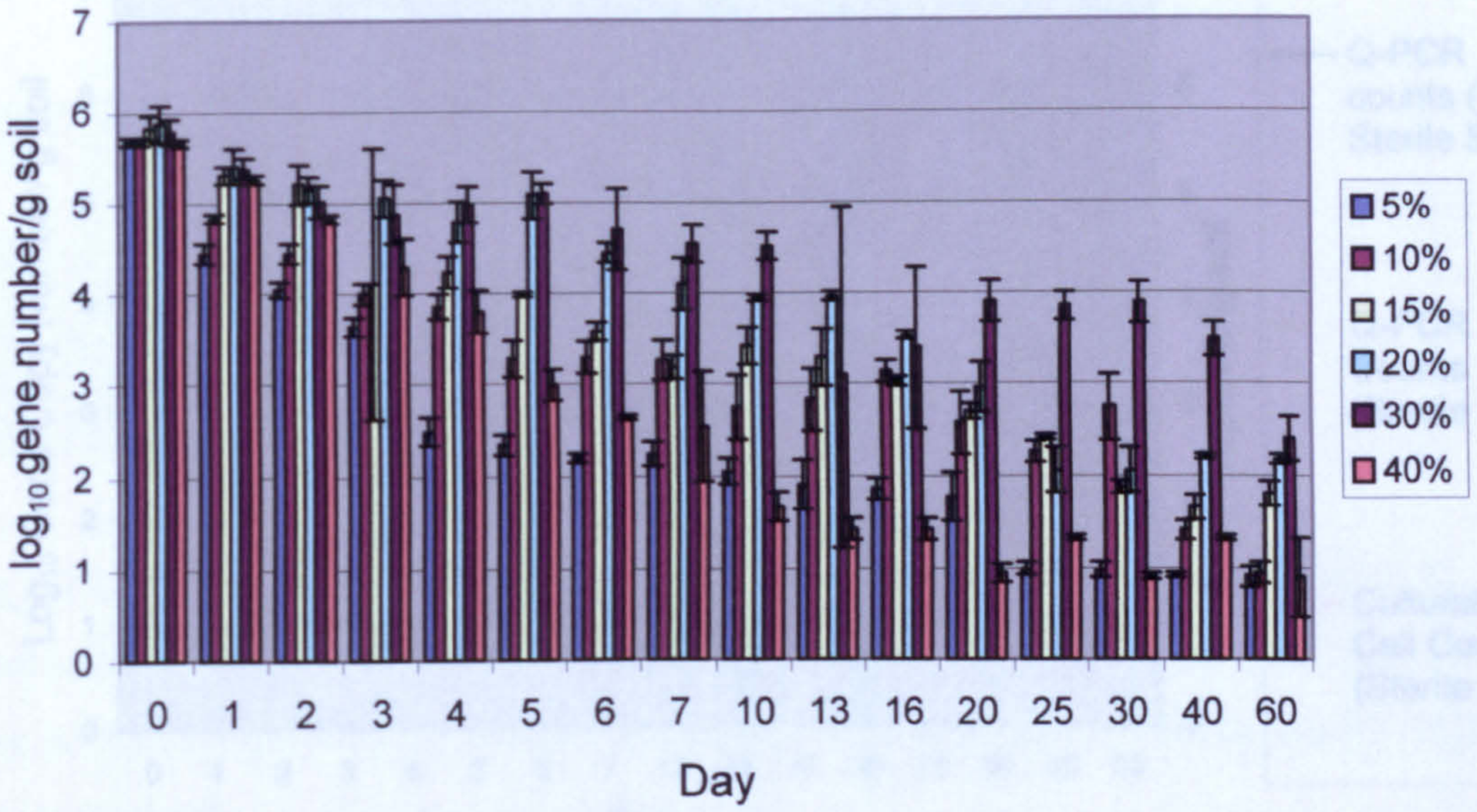


Fig. 3.23 Survival of *M. bovis* BCG in non-sterile soil microcosms at different soil wetting levels (PCR gene count data).

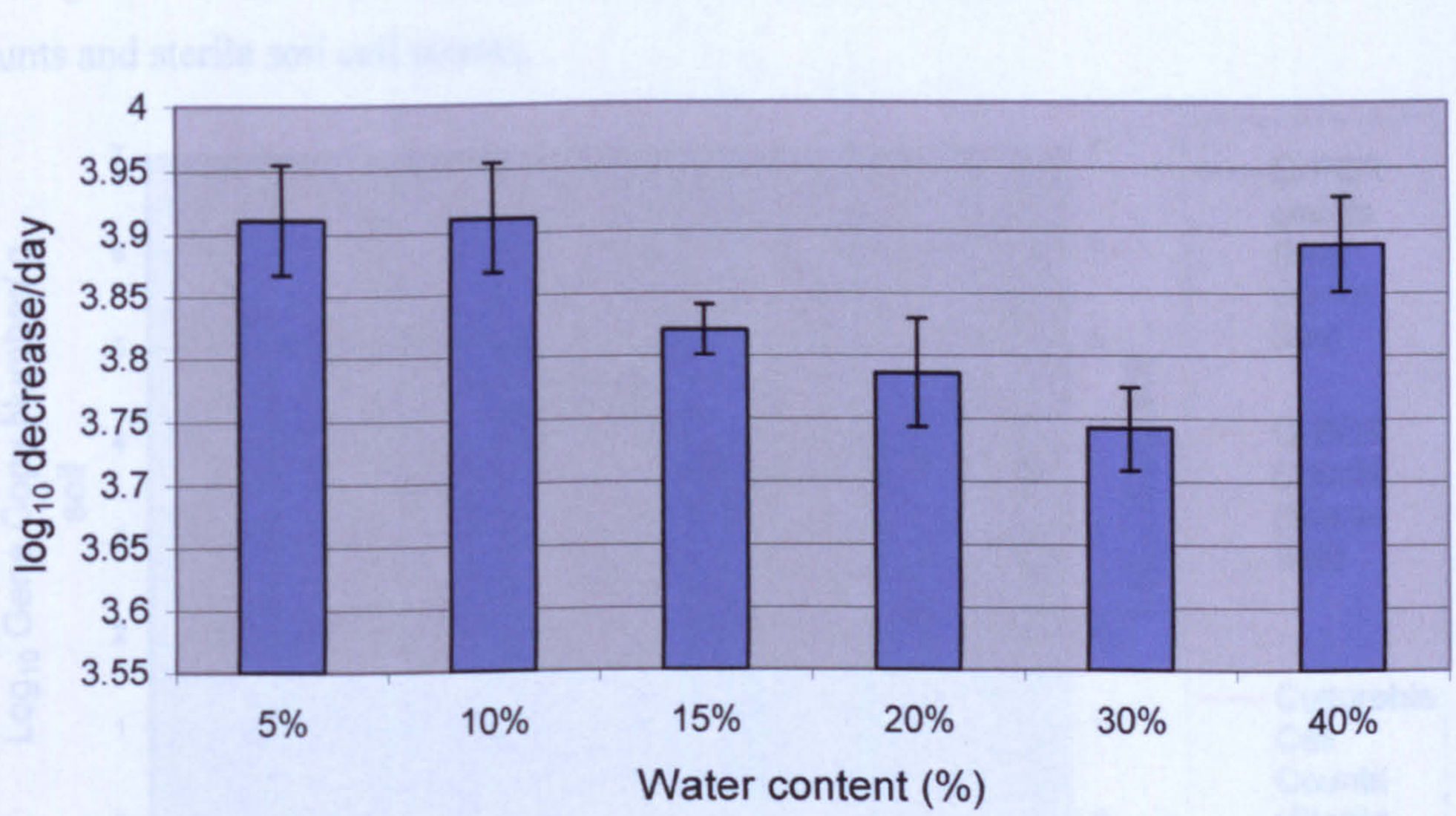


Fig. 3.24 Decrease in gene copy number per day rates for *M. bovis* BCG in non-sterile soil microcosms at different soil wetting levels (PCR gene count data).

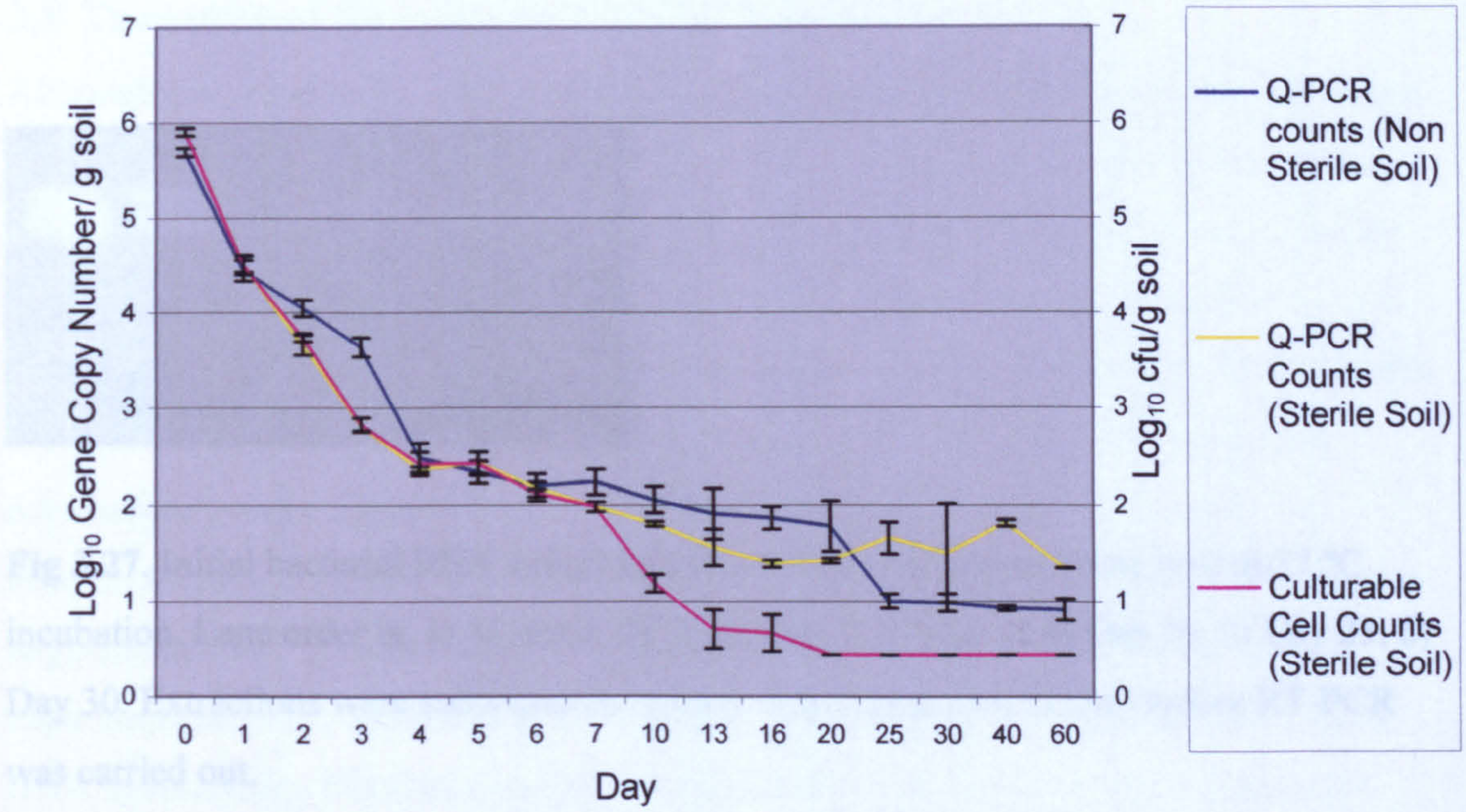


Fig. 3.25. Combined microcosm data for survival of *M. bovis* BCG incubated at a 5 % wetting level. Three experiments are shown, non-sterile soil gene counts, sterile soil gene counts and sterile soil cell counts.

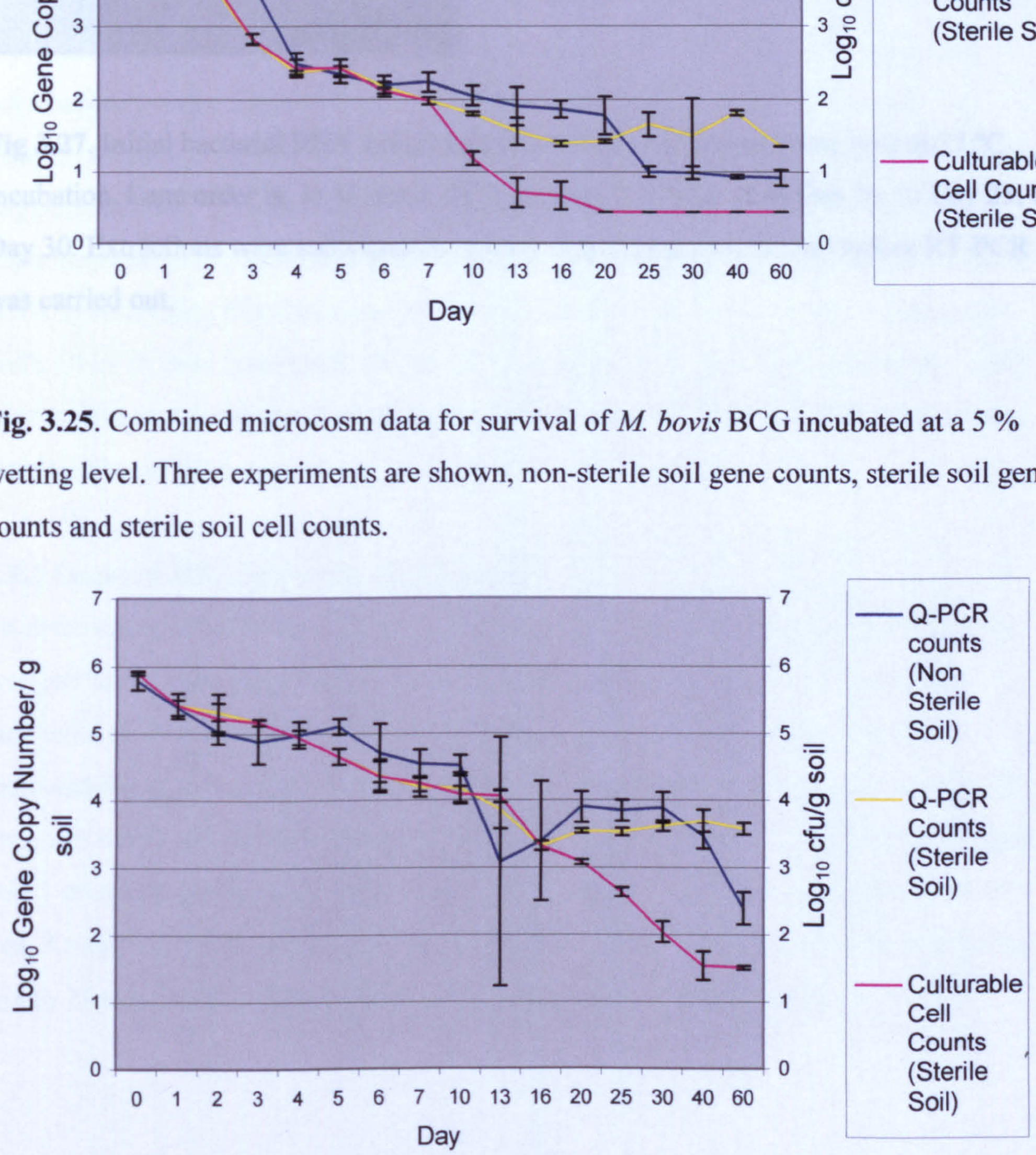


Fig. 3.26. Combined microcosm data for survival of *M. bovis* BCG incubated at 30 % wetting level. Three experiments are shown, non-sterile soil gene counts, sterile soil gene counts and sterile soil cell counts.

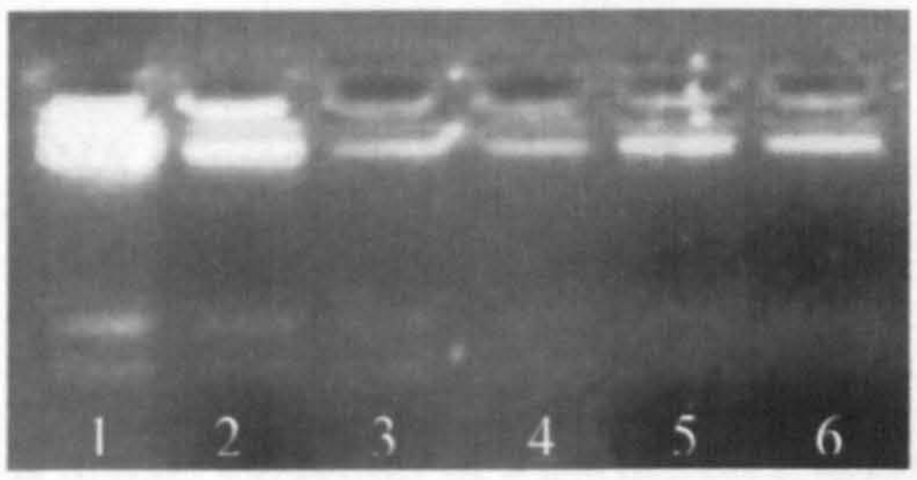


Fig 3.27. Initial bacterial RNA extractions from sterile soil microcosms held at 37 °C incubation. Lane order is, 1) *M. bovis* BCG, 2) Day 7, 3) Day 15 4) Day 20, 5) Day 25, 6) Day 30. Extractions were subsequently treated with RNase-free DNase before RT-PCR was carried out.

3.8 Turnover of *M. bovis* BCG DNA in soil

Although the presence of DNA for specific target sequences in soil community DNA can be a good indication of the presence of viable cells, it is by no means guaranteed. Some DNA sequences can remain for long periods of time outside of the cell environment, by binding to clay particulates in soils (Trevors, 1996). This protects the DNA from degradation by extracellular nucleases (Demanèche *et al.*, 2001). This is of interest in that it means detection of a particular gene in total community DNA from soil, does not automatically mean intact live cells associated with the gene are present in the soil sample. As such the previous PCR data presented in this thesis gives survival of the gene targets in soil and the survival of the intact organisms could only be inferred. An experiment therefore needed to be devised to determine the longevity of *M. bovis* BCG DNA in soils using DNA in a variety of states. DNA in live cells, DNA in dead intact cells, DNA in dead lysed cells, and free chromosomal DNA were inoculated into soil microcosms and presence of a target gene *mpb70*, as an indicator of DNA persistence, monitored over time.

3.8.1 Decay of DNA from intact cells in soil

To determine the turnover of DNA in intact live cells, 1g non-sterile soil microcosms were set up an inoculated with 10^6 cells of *M. bovis* BCG. Microcosms were then incubated at 10°C and 30°C, with samples taken every day for 20 days. DNA was extracted from microcosms at each time point and PCR carried out targeted to *mpb70.* Products were electrophoresed and gels analysed using TotalLab 1d gel analysis program. Day 1 products were calculated as being 100 % with further days calculated as a % of this. Results are given in Fig 3.28 By day 11 levels of DNA had fallen to 48 % at 10°C, and to 71 % at 30°C. These levels then remained constant until day 20.

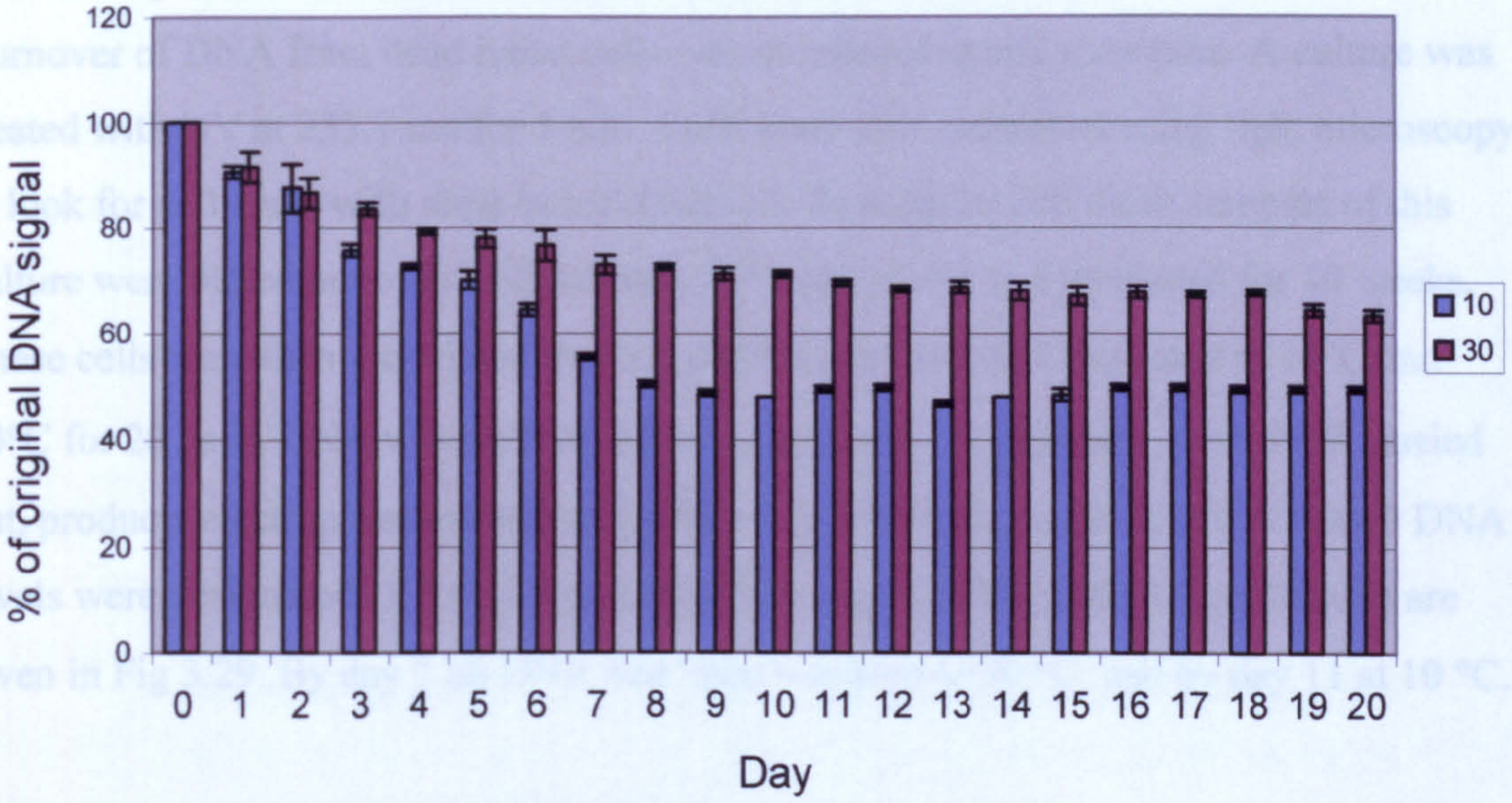


Fig. 3.28 Presence of DNA from live *M. bovis* BCG cells in soil microcosms incubated at 10°C and 30°C.

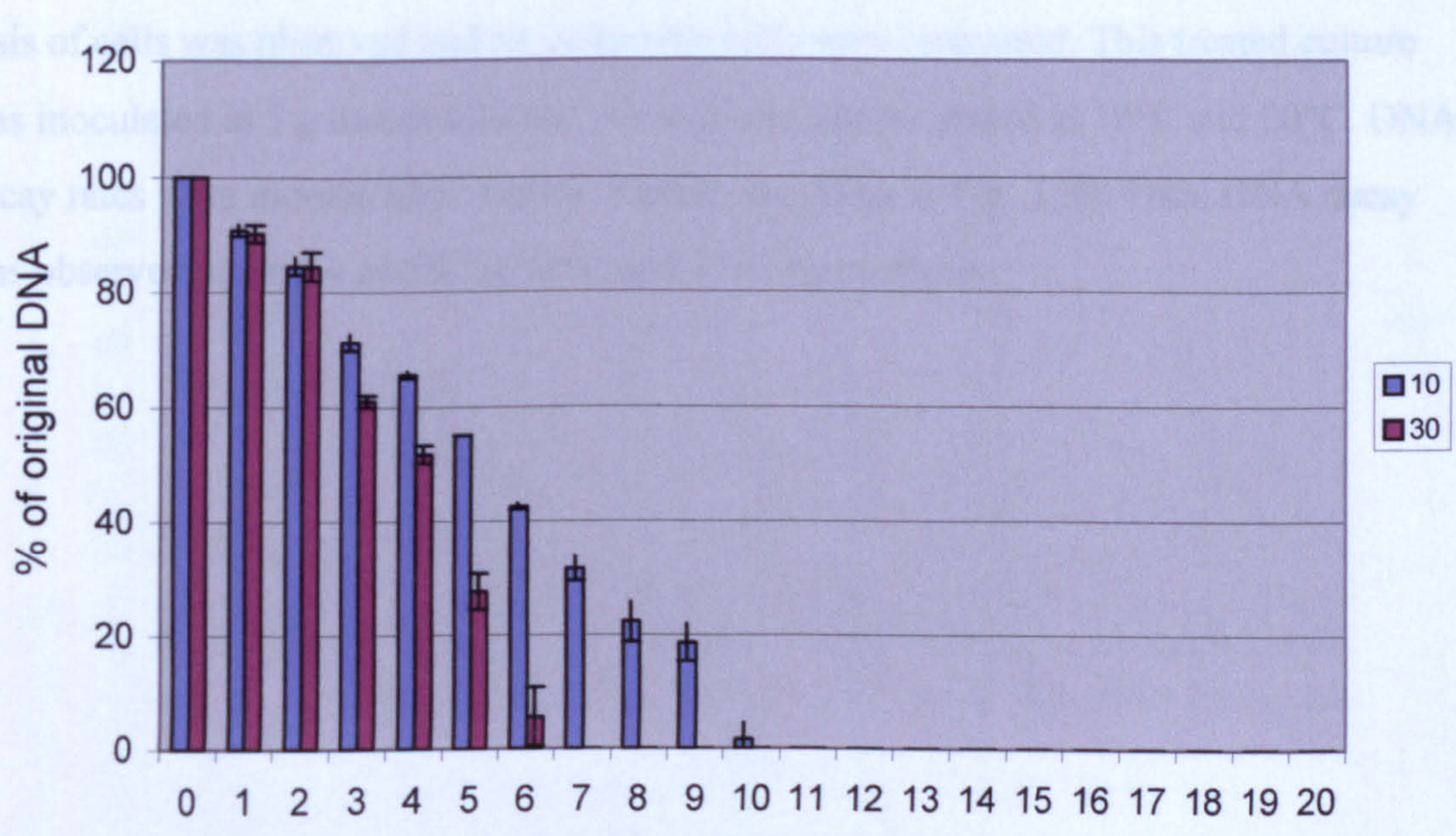


Fig. 3.29 Presence of DNA from dead intact *M. bovis* BCG cells in soil microcosms incubated at 10°C and 30°C.

3.8.2 Decay of DNA from dead intact cells in soil

Turnover of DNA from dead intact cells was monitored in soil over time. A culture was treated with UV at 253.7 nm for 5 min. Cells were then examined using light microscopy to look for cell lysis, with none being observed. To monitor cell death samples of this culture were plated out onto Middlebrook 7H9 agar plates and incubated for 10 weeks. These cells were then inoculated into 1 g soil microcosms and incubated at 10°C and 30°C for 20 days. DNA was extracted from microcosms at each day point, PCR carried out, products electrophoresed and analysed using TotalLab. Again as before, day 0 DNA levels were designated as 100 % and further day points as % of this value. Results are given in Fig 3.29. By day 7 all DNA had been removed at 30 °C, and by day 11 at 10 °C.

3.8.3 Decay of DNA from dead lysed cells in soil

Cells in a culture of *M. bovis* BCG were lysed by heating at 100°C for 10 min. Cell lysis was observed using light microscopy and viability of cells monitored by plating the culture onto Middlebrook 7H8 agar plates and incubating at 30°C for 10 weeks. Total lysis of cells was observed and no culturable cells were recovered. This treated culture was inoculated in 1 g non-sterile soil microcosms and incubated at 10°C and 30°C. DNA decay rates were monitored as before. Results are given in Fig. 3.30. Total DNA decay was observed at days 4 and 6 for 10°C and 30°C respectively.

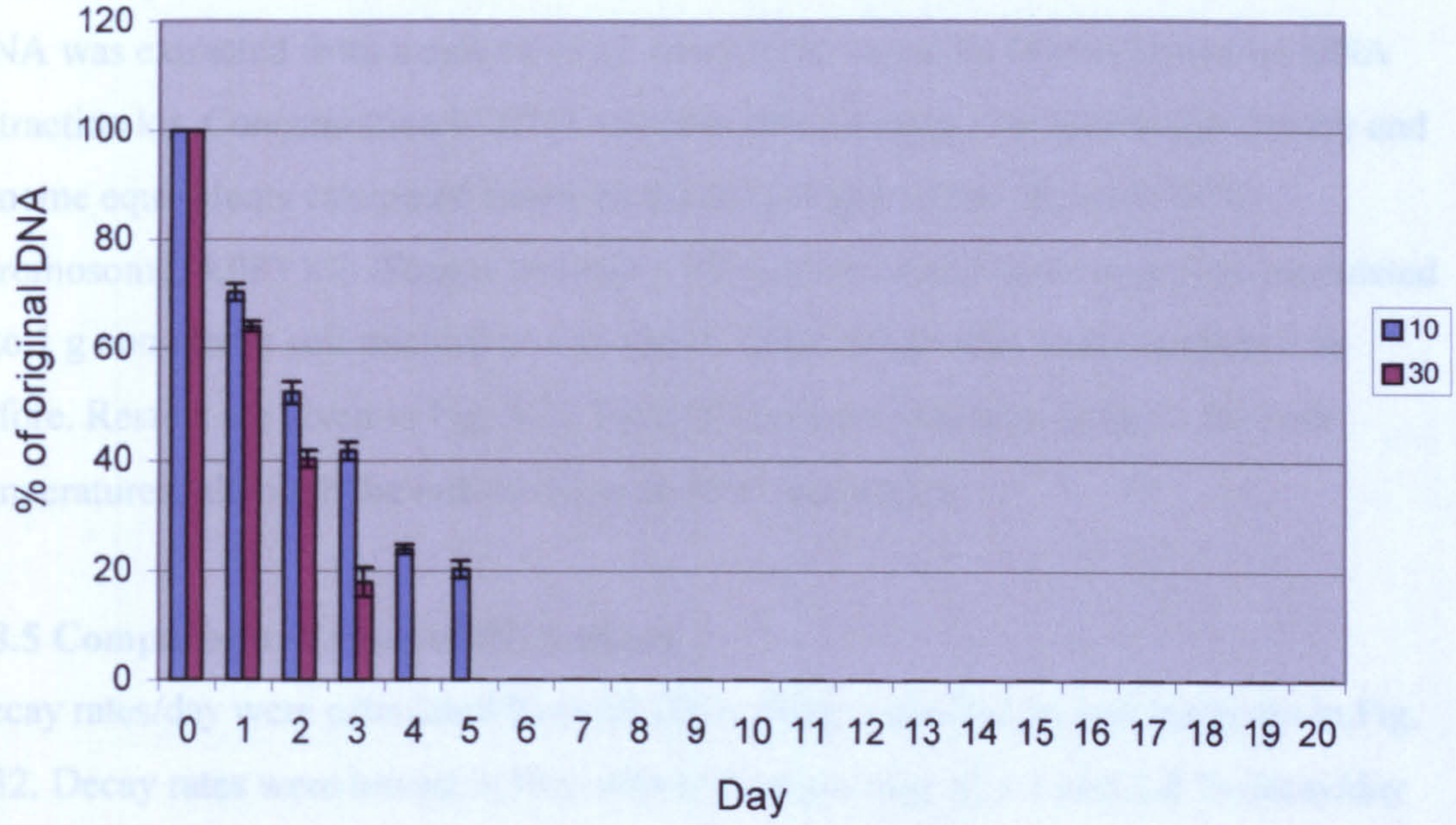


Fig. 3.30 Presence of DNA from dead lysed *M. bovis* BCG cells in soil microcosms incubated at 10°C and 30°C.

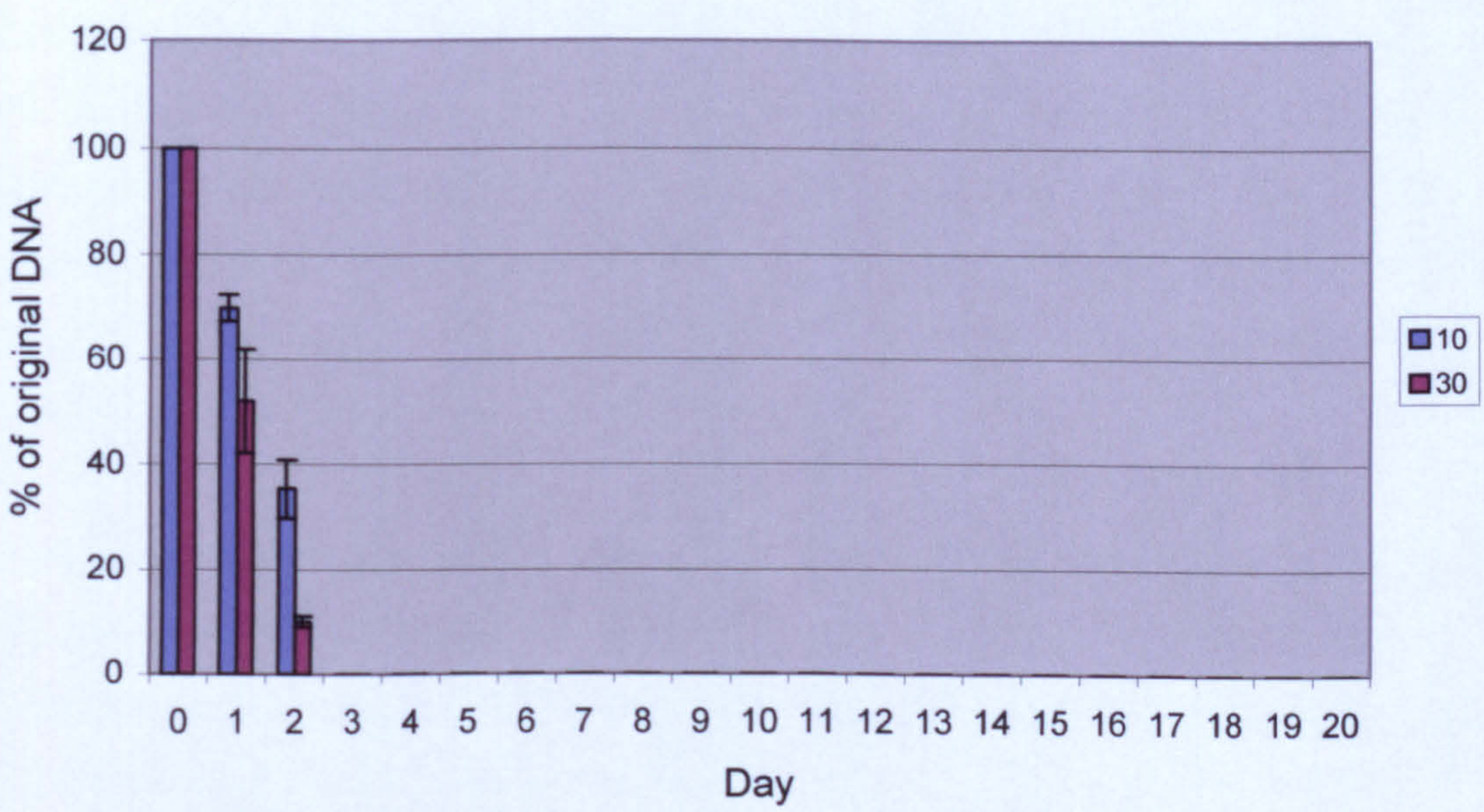


Fig 3.31 Presence of DNA extracted from *M. bovis* BCG cells in soil microcosms incubated at 10°C and 30°C.

3.8.4 Decay of free DNA in soil

DNA was extracted from a culture of *M. bovis* BCG using the Mobio microbial DNA extraction kit. Concentration of DNA was determined using UV spectrophotometry and genome equivalents calculated based on the total length of the *M. bovis* BCG chromosome, 4,083 Mb (Sanger Institute). 10^8 genome equivalents were then inoculated into 1 g non-sterile soil microcosms as above. DNA decay rates were monitored as before. Results are given in Fig. 3.31 Total DNA decay was seen at day 3 for both temperatures, although the rate of decay at 30°C was higher.

3.8.5 Comparison of rates of DNA decay

Decay rates/day were calculated from all DNA decay experiments and are given in Fig. 3.32. Decay rates were lowest in live cells with an average of 2.5 and 2.8 % decay/day for 10°C and 30°C respectively. Decay rates next highest in dead intact cells, then dead lysed cells and highest decay rates were seen in free DNA with rates of 21.5% and 30 % decay/day at 10°C and 30°C respectively.

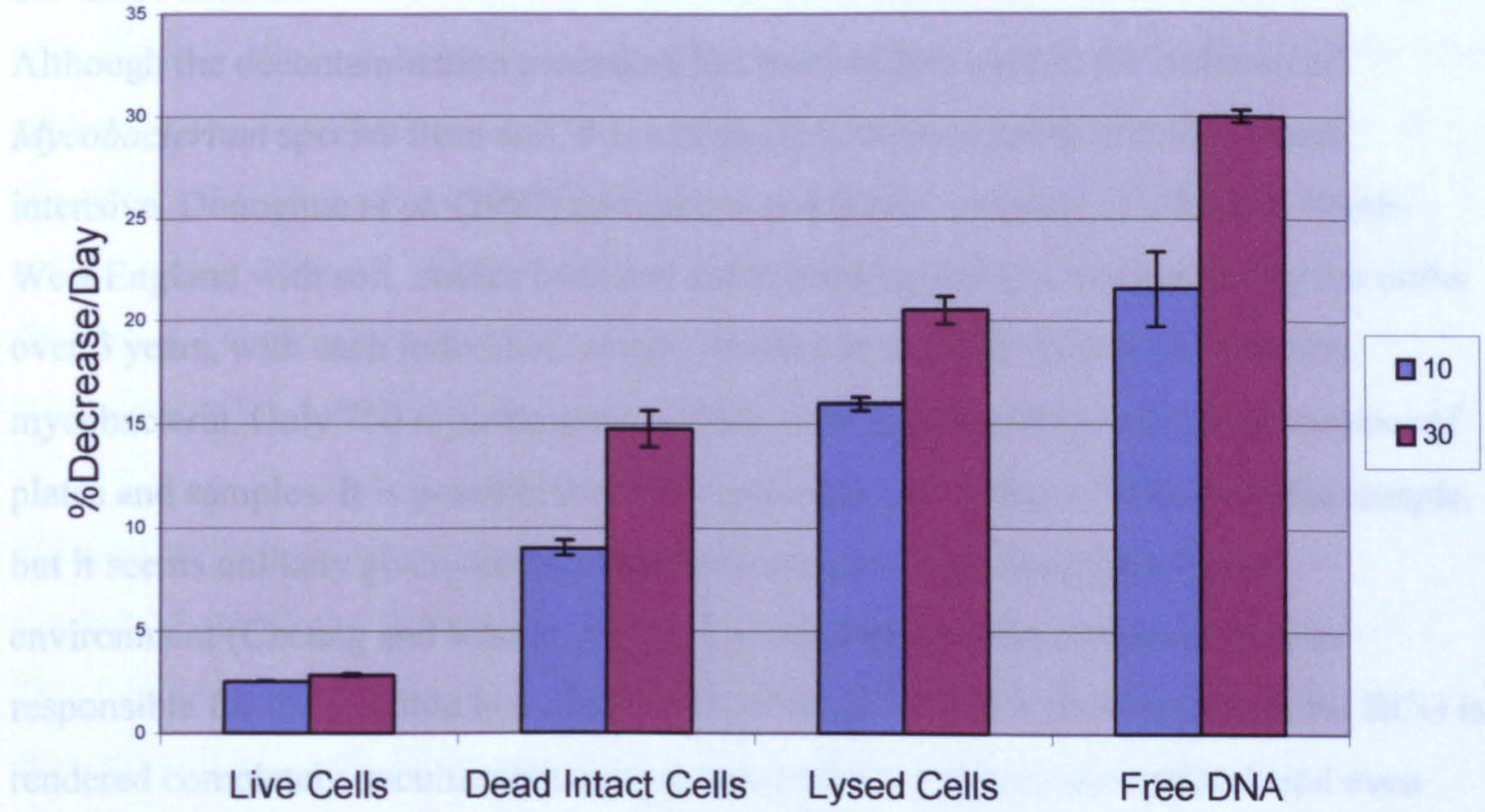


Fig. 3.32 Decrease of DNA per day rates from four different sources in soil microcosms incubated at 10°C and 30°C.

3.9 Discussion

Although the decontamination procedure has been widely used in the isolation of *Mycobacterium* species from soil, it is extremely time consuming and also labour intensive. Donoghue *et al.* (1997) carried out systematic sampling of a farm in South-West England with soil, stream beds and cattle drinking troughs sampled every 3 months over 3 years, with each individual sample resulted in over 100 plates for culturing mycobacteria. Only 750 mycobacteria isolates were detected despite the huge number of plates and samples. It is possible that this represents the number of species in the sample, but it seems unlikely given that mycobacteria are seemingly ubiquitous in the environment (Cheung and Kinkle, 2001). The decontamination procedure may be responsible for the possible low numbers. Results given in 3.3 show that *M. bovis* BCG is rendered completely unculturable using a standard decontamination method, and even more robust species e.g. *M. phlei* are drastically reduced in numbers. This may of course be a product of the specific method employed, however even when multiple methods are employed, isolation numbers still appear to be low. Iivanainen *et al.* (1999), sampled indoor swimming pool water using four different methods, with none giving more than a 46 % success rate in isolation of *Mycobacterium* species from the samples. The use of decontamination is therefore a compromise between removal of non-mycobacteria and the isolation of mycobacteria from a sample. To overcome the associated problems it was decided to employ molecular methods to detect mycobacteria specifically *M. bovis* in environmental samples.

To produce a specific and accurate method of detecting *M. bovis* strains and sub-strains in the soil environment, three target genes were chosen which had been proved to be specific for the *M. tuberculosis/M. bovis* complex. These three genes *mpb70, mpb64* and *esat6* are present in varying strains and are all present in *M. bovis* and *M. bovis* BCG Pastuer. The use of these genes as targets allowed rapid PCR detection of *M. bovis/M. tuberculosis* in a given sample. Testing of several species indicates that the antigen genes are only found in *M. bovis/M. tuberculosis* with the exception of *esat6*. The primers used to detect this gene gave some non-specific banding in *Streptomyces coelicolor*, but not in any other species tested. Also using primer sets for the three genes in total community

DNA extracted from different soils *esat6* primers gave multiple non-specific bands in all soils tested whereas *mpb64* and *mpb70* did not. This leads to the possibility that either the primers are randomly priming in soil DNA or that there are *esat6*-like genes present in the environment. Indeed Pallen (2002) postulates that *esat6* may be part of a secretion system widespread in Gram-positive bacteria and indeed that some homologues can be detected in the *Streptomyces coelicolor* genome. Due to this lack of specificity this primer set was not used further.

This research aimed to detect mycobateria in soil, and to attempt to quantify cell numbers. LightCycler technology was employed which ultimately proved unsuccessful. This lack of accurate quantitation may be due to humic acid contaminants carried over from the soil when the DNA was extracted. Although quantitative PCR has been successfully used on DNA extracted from soil (Kolb *et al.*, 2003) fluorescence quenching and auto-fluorescence of a sample has been noted previously when using fluorescent quantitative PCR (using both TaqMan and LightCycler technologies) (Stults *et al.*, 2001). To overcome this, dilution of the DNA sample is necessary to reduce the humic contamination, obviously this also dilutes the DNA and severely raised the limits of detection of the system. Stubner (2002), found that the limit of detection of a specific PCR designed to target *Desulfotomaculum* species' 16S rRNA sequences rose from 10^2 targets in chromosomal DNA to 10^6 targets in spiked soil DNA, due to the necessary dilutions. As noted in Fig 3.6, the melting curve did not give an accurate measure of product formation with the majority of fluorescence present as non-specific products. This was also noted by Stubner's group who monitored product accuracy through gel electrophoresis, as the melting curve did not give a distinct peak when soil DNA was examined.

As Real-Time PCR was unsuccessful, a new method was devised to quantify PCR products directly from agarose gels. This new method relied on the use of PCR standards, in this case a dilution series of *M. bovis* BCG inoculated into soil and total DNA extracted using the same method as unknown samples. These standards would therefore

have the same extraction efficacies, and the same PCR inhibitors carried over as unknown samples.

This new method was employed to monitor survival of *M. bovis* BCG in several microcosm experiments, alongside culturable cell counts. *M. bovis* BCG was inoculated into 1 g soil microcosms and monitored over time in the first instance under varying wetting levels. From these experiments overall BCG survives at a higher level the higher the water content of a soil is up to a maximum of 30 % (matric potential of greater than -10 KPa). Over this the levels of survival decrease. Levels over 30 % would be waterlogged conditions severely limiting oxygen availability to the bacteria, and rendering the microcosms increasingly anoxic. As mycobacteria are aerobic organisms, they would be severely limited in these conditions. This is supported by culturable counts where levels of cells drop at a higher rate at 40 % water content. As 40 % water content equates to a matric potential of -2 Kpa this means the soil is reaching saturation, and the distance that oxygen molecules have to diffuse across is at its maximum, reducing oxygen bioavailability. Conversely, extremely dry conditions i.e. 5% water levels, (matric potential of less than –1500 KPa) lowered the levels of nutrients available to the organisms as the lack of water meant diffusible molecules became increasingly less available for uptake into the cell. At all water levels culturable cells were no longer detected after 60 days, however DNA data gave persistence of DNA from BCG, in some cases, up to 18 months after inoculation. This raised the question if this result was due to persistence of cells or to persistence of DNA. To answer this, microcosms were devised to determine the persistence of DNA from a variety of sources in soil. These experiments showed that BCG DNA only survives for significant periods of time when present in live intact cells. Free DNA cannot be detected after only 3 days, with DNA from dead intact cells, and lysed cells undetectable between 5 and 10 days after inoculation. This would suggest that when PCR products are detected in soil specific for BCG, they come from a live viable (but not necessarily culturable) cell, or at least a cell that was viable for between 5 and 10 days previously.

The phenomena of cells being viable but non-culturable is widely recognized, with many bacterial species possessing the ability to enter a dormant state e.g. *Salmonella* species (Turpin *et al.*, 1993; Asakura *et al.*, 2002a; Asakura *et al.*, 2002b), *Enterococcus* species (Heim *et al.*, 2002), and *Vibrio* species (Weichart and Kjelleberg, 1996). *M. tuberculosis* can enter long periods of dormancy triggered by a gradual decrease in oxygen levels (Cunningham and Spreadbury, 1998). *M. tuberculosis* can survive for many years in this state after an initial infection, and can suddenly become viable again if the host becomes immuno-compromised in some way (Zhang *et al.*, 2001). As *M. bovis* is extremely closely related to *M. tuberculosis* it is possible that this organism enters dormancy in a similar manner. Boon and Dick (2002) discovered that *M. bovis* BCG entered this dormancy state with specific genes up-regulated e.g. *dosR*, when increasing hypoxia occurred. The results presented in this thesis suggest that BCG survived well and remained culturable at up to 30% water levels in soil, but over that water level culturability decreased and dormancy was triggered. Hypoxia may not be the only trigger for dormancy however, as all wetting levels experiments exhibited a decrease in culturability coupled with a persistence of DNA, other triggers could possibly include nutrient deprivation, and toxicity build up.

Experiments to determine survival of BCG at different temperatures suggest that BCG survived at a higher level and remained culturable for longer at higher temperatures. As BCG was derived from an intracellular pathogen, its optimal growth temperature is adapted to be 37°C, and it was expected that this temperature would give the greatest survival. Although culturability decreases at all temperatures, persistence of DNA was found at up to 18 months after inoculation. As there were differences in this rate of decrease, it suggests, as mentioned previously, that water content and hypoxia may not be the sole trigger for dormancy in BCG, as all these experiments were carried out at a constant level of 15 % w/v water content.

These experiments were carried out in sterile soil, and were then repeated in non-sterile soil. This had a marked effect on survival of BCG. Comparison graphs (Figs 3.24 and 3.25) show at 5% and 30% wetting levels, as expected, the survival of cells was lower in

non sterile soil than in sterile soil, due to the presence of indigenous bacteria in the soil more suitably adapted to a soil environment. BCG has huge deletions introduced in the genome, when the strain was first created (Brosch *et al.*, 2001), which may have removed genes involved in nutrient acquisition and also stress-related genes. However as seen in Figs 3.16 and 3.17 at 37°C survival in non-sterile and sterile soil was indistinguishable. This may be due to the increased growth rate of BCG at this temperature, and the lack of ability for some indigenous soil organisms to survival at this temperature. At 4°C survival of BCG is greater in non-sterile soil than in sterile soil. Indigenous soil bacteria would be better adapted to survive and compete at this temperature, whereas BCG would not, so growth rates and competitiveness do not appear to be affecting this survival. It could be another trigger for dormancy i.e. as temperatures lower, dormancy gene expression is triggered. Cold shock has been shown to increase the expression of *sigF* a gene associated with the regulation of the dormancy response in *M. tuberculosis* (Michele *et al.*, 1999). A second option is that a decreased fluidity in cell wall and membrane structure could protect the BCG cells against uptake of toxins or attack from extracellular nucleases and proteases. The components of the mycobacteria cell wall and membrane have high melting temperatures and have low fluidity, and at low temperatures the membrane is almost static (Liu *et al.*, 1995). Experiments have shown that diffusion of molecules across the membrane is extremely low at temperatures below 15°C (Liu *et al.*, 1996).

As can be seen in all these experiments some day sampling points show large standard deviation error bars compared to the majority. This can be explained by clumping of the cells. Mycobacteria are extremely hydrophobic and clump quite readily during culturing (Leopold and Fischer, 1993). Although shaking was employed along with the use of surfactants, total dispersion of the culture was not possible, therefore some slight clumping of cells may have occurred which could have skewed these individual results.

M. bovis BCG has been shown to survive in a viable state for long periods of time optimally at 37°C and at 30 % (v/w) soil water content. These results should be carefully considered in any use of BCG in the vaccination of badgers. Careful monitoring of BCG

in feed baits and its possible dissemination into soils should be carried out as part of any further field trials of vaccination. This is of particular concern in areas where badger sets are in close proximity to cattle fields, as the risk of cattle being exposed to the vaccine and therefore becoming PPD test reactors, is of concern to the current lack of a readily available system for determining differences between infected and vaccinated cattle.

Chapter 4: Diversity of *Mycobacterium* species in soil

4.1 Introduction

The classification of the *Mycobacterium* genus is extremely complex as described in **Chapter 1.** The simplest groupings are that the genus can be divided into two groups; fast (or rapid) growers and slow growers (Stahl and Urbance, 1990), and into tuberculous mycobacteria and non-tuberculous mycobacteria (Cook *et al.*, 2003). The majority of fast growers are soil saprophytic organisms e.g. *M. fortuitum* (Kirschner *et al.*, 1992), and *Mycobacterium hiberniae* (Kazda *et al.*, 1993), and typically can be cultured in the laboratory in less than seven days. The slow growing group (defined as having a culture time of greater than seven days) contains soil saprophytes, but also contains species which have the capacity to be highly pathogenic e.g. *M. bovis* and *M. avium (*Ji *et al.*, 1994). The classification and phylogeny of the mycobacteria has been widely studied initially due the interest in species as pathogens, but latterly as certain fast-growing species have been identified as playing important roles in the biodegradation of some xenobiotic compounds in the environment (Sutherland *et al.*, 2002; Margesin *et al.*, 2003).

Initially mycobacteria were classified biochemically and phenotypically, with the basic groups defined using such criteria as colony morphology, pigment production, host range and growth rate Although many individual species were successfully identified using these methods, several have seen changes in nomenclature with the advent of molecular tools. *M. avium* and *M. paratuberculosis* have been reclassified as *M. avium* subsp. *avium* and M. *avium* subsp. *paratuberculosis* respectively (Thorel *et al.*, 1990) A secondary problem with identifying species using these methods, especially in a clinical environment, was the time taken to gain results. The use of these biochemical and phenotypic properties also has problems in the identification of environmental strains of mycobacteria, where these strains show differing properties from the established type-strain, i.e. growth temperature, substrate hydrolysis and pigmentation (da Silva Rocha *et al.*, 1999).

With the advent of molecular analysis of nucleic acid and protein content of cells, several new methods to identify mycobacteria in both environmental and clinical samples were developed (Tortoli, 2003). Protein and lipid analysis has been used to type new clinical and also to create phylogenies for type strains of mycobacteria (Koukila-Kahkola *et al.*, 1995; Springer *et al.*, 1995;). The majority of research has focused on differentiating mycobacteria using mycolic acid analysis. This molecule is the predominant component of the thick cell wall and differentiation is derived from studying both chain length of the mycolic acids and also the compostion using, for example, HPLC (Brown *et al.*, 1999; Butler and Guthertz, 2001). Also studied is the lipid content of cells and specifically the cell wall using HPLC and gas chromotography (Koukila-Kahkola *et al.*, 2000; Torkko *et al.*, 2003). A polyphasic approach to classify mycobacteria is regarded as the most reliable (Wayne *et al.*, 1996), however it is of limited use in environmental samples. The techniques rely on the strains/species being investigated to be present in high numbers in order for enough material to be extracted. Secondly there is a requirement for a culture of a single species/strain (or a limited number of species/strains) which would not be present in the majority of environmental samples.

Analysis of genes present in *Mycobacterium* spp. is also used, mainly analysis of the 16S rRNA encoding genes of species, and comparison of sequences using phylogenetic analysis (Ji *et al.*, 1994; Wang *et al.*, 1995; Cloud *et al.*, 2002). Providing a sufficient quantity of cell matter is present DNA can easily be extracted and analysed. This can be a problem however in identifying species in some cases where cell numbers are low, and is usually accompanied by a cell extraction and growth step of the organisms before DNA extraction. The majority of mycobacteria species have had their 16S rRNA encoding genes sequenced (Genbank) and using this information the species groupings obtained through phenotypic methods have been proven to be mostly correct (Pitulle *et al.*, 1992). However, some anomalies emerged possibly due to the lack of genetic diversity among 16S sequences apparent in the genus (Clayton *et al.*, 1995). It is for example impossible to distinguish *M. bovis* from *M. tuberculosis* using 16S rRNA analysis as the genes are identical (Hughes *et al.*, 1993), as is the separation of *M. avium* and *M.avium* subsp. *paratuberculosis* strains (Bannantine *et al.*, 2003), despite the differences in infectivity,

growth rate and host range. Also environmental strains which have differing biochemical properties, due to niche adaptation, may have identical 16S rRNA sequences due to the evolutionary rate of the molecule (Boddinghaus *et al.*, 1990). Several other genetic markers have been employed to discriminate between mycobacteria, including the housekeeping gene *recA (*Blackwood *et al.*, 2000*)*, the 65 KDa heat shock protein encoding gene, *hsp65* (de Magelhaes *et al.*, 2002) the 23S rRNA gene (Kurabachew *et al.*, 2003), the internal transcribed spacer (ITS) (Hamid *et al.*, 2002) and *gyrB* (Niemann *et al.*, 2000). These all have their disadvantages however, due to the similarity of some species, and also due to the lack of sequence information available for these markers, when compared to 16S rRNA data. Approximately 40 sequences for *gyrB*, and 100 for *hsp65* are present in Genbank, compared to over 1000 individual sequences for 16S rRNA. Although 40 and 100 sequences may appear to be an adequate number for coverage of the genus, many of these are partial sequences and/or multiple strain sequences of one particular species, rather than unique sequences for individual species.

The use of molecular markers in the identification of environmental mycobacteria means that individual species and also communities can be analysed directly from environmental samples, e.g. soil, without the need for a cell extraction and culture step. As stated previously in **Chapter 3**, this is problematic due to time considerations, but also due to the nature of the culture techniques employed. These techniques have to remove the majority of non-mycobacteria species from the sample, to stop the contamination of culture media, which would otherwise occur. These methods therefore employ several harsh chemical additions, and may have some effect on the numbers and the diversity of the mycobacteria extracted.

Several studies have focused on using 16S rRNA analysis to identify mycobacteria in soil samples, and have revealed the wide variation present in the environment. Mendum *et al,* (2000) used PCR analysis on total community DNA extracted from several soil samples in areas used for wheat growth. Only a small number of unique sequences were identified, although several formed distinct clades from type strain sequences. There is also a problem in using these techniques in total community DNA, in that some PCR

primers may be inherently biased towards some species, particularly where some species may be present in greater numbers than others, or where primers have been designed with mis-matches (usually unavoidable when creating genus-specific primers) and so the resulting data may be skewed. It is therefore important to not rely on a single primer set when analyzing diversity in total community DNA. Taking the advantages and disadvantages of each method into account, it was decided to use analysis of 16S rRNA in this research to determine the diversity present in selected soil samples, due to the number of sequences available for comparison, the ease at which sequences can be differentiated through sequencing, and the ability to specifically detect mycobacterial 16S rRNA and 16SrDNA sequences.

As with all sampling and detection regimes for determining the diversity of a target group, their validation is extremely important. No sampling can be exhaustive and so statistics need to be employed, in order to discover the efficacy of the experiment. Several statistical analyses are available for estimating abundance of species in a sample compared to the observed quantities. The most widely used statistic in analysis of species abundance is rarefaction (curve) analysis (Coleman, 1981). This estimates species richness over different samples that are pooled. As more samples are added to the calculation the resulting curve levels off as each individual sample contains fewer and fewer unique species. Obviously the more diverse the samples are, the more samples are needed in order to produce this result. This statistic can therefore be used to determine if enough samples have been tested to accurately represent diversity. This analysis has been superceded by Coleman rarefaction curve analysis (Coleman *et al.*, 1982) which is based on the same algorithms, but is computationally more accurate. Again the resulting curve levels off as the number of uniques decreases. This method can also be used to compare samples as observed diversity can be plotted alongside estimated diversity. The lower the observed curve is compared to the estimated curve, the more diverse a sample is. The closer the curves are, the less diverse. Other analyses can be used to determine diversity coverage. The most predominant ones are ACE (Abundance-based Coverage Estimator of species richness) (Colwell and Coddington, 1994), Jack1 (First-order Jackknife richness estimator) and Bootstrap (Bootstrap richness estimator) (Smith & van Belle,

1984). Each of these uses different matrix-based computational analyses and should be used in conjunction in order to gain accurate estimations. It is important with any analysis to compare several methods, as single statistical analysis can give inaccurate results. For example MMruns (Michaelis-Menten richness estimator: estimators averaged over randomizations (runs) (Raaijmakers, 1987), can greatly over-estimate diversity in highly diverse samples due to the way the analysis averages the estimators. Multiple randomisations are used and one estimator, which has a high value, can be falsely reproduced across each randomisation. In this research several estimators were used and results compared

The analysis of the mycobacterial population in this study is important due to the impact on the rates of bovine TB and also the identification of animals infected with the disease. It is thought that some fast growers can indirectly affect an individual's susceptibility to infection by *M. bovis* and the following detection of infection (Buddle *et al.*, 2002). In the case of bovine tuberculosis the exact effect is unknown although studies have shown several possible reasons (Pollock *et al.*, 2002). Certain species have similar antigenic properties to *M. bovis* e.g. *M. hiberniae* (Jungersen *et al.*, 2002). This can lead to two outcomes if an individual animal comes into contact with the latter species. Firstly the individual raises an immune response to the antigenic factors, and when subsequent testing with *M. bovis* PPD (purified protein derivative) is carried out, this response is detected and mistakenly diagnosed as a case of bovine tuberculosis (Auer and Schleehauf, 1988). There have been several cases where animals giving a positive test result have shown no evidence of *M. bovis* infection following further investigation. (DEFRA, 2003) Secondly, this antigenic response to species other than *M. bovis* can immunologically prime an animal. This means if the animal is infected with *M. bovis* following this exposure, the immune response will be much higher and more immediate, resulting in greater tissue damage than an infection without prior exposure (The majority of tissue damage being caused by the immune response rather than the infecting organisms) (Howard *et al.*, 2002). Finally prior exposure to indigenous environmental mycobacteria can severely lower the efficacy of vaccination of individuals with *M. bovis* BCG. It is believed that the immune response is targeted immediately to the vaccine after

prior exposure reducing the vaccine uptake, as *M. bovis* BCG needs to replicate several times in the host for effective vaccination to take place (Suazo *et al.*, 2003).

4.2 Aims

The aim of this research was to analyse 16S rRNA encoding genes in total community DNA from soils in fields to determine the detectable diversity of the mycobacteria population in soil from a farm in Southern Ireland. Sample sites were chosen with and without exposure to *M. bovis* infected animals to allow comparison of populations (including possible presence of *M. bovis*) in areas of infection and non-infection with bovine TB. Different statistical analyses of data sets were studied, to determine if the sampling regime and molecular detection techniques gave a true representation of the diversity of the mycobacteria population.

To use the methods developed for 16S rRNA analysis to determine the presence, or absence of *M. bovis* BCG 16S rRNA sequences in soil microcosms using DNA and RNA extraction followed by PCR and RT-PCR. This will determine the viability of the cells over time under different environmental conditions (refer to **Chapter 3**)

4.3 Diversity of the *Mycobacterium* genus

In order to facilitate the design of PCR primers targeted to the *Mycobacterium* genus, a phylogenetic tree was constructed using representatives from the different groups of the mycobacteria. Sequences of 16S rRNA encoding genes were obtained from Genbank and aligned using Bioedit (refer to **Section 2.13.1**). Over 200 sequences from species, strains and uncultured bacteria were included, with only those sequences over 1000 bp in length being retained. Shorter sequences were discarded and where possible gaps in the alignment were removed. When comparing sequences from slow and fast growers the 451-482 long helix insertion (or deletion) was counted as a single evolutionary event, with the gap in the fast grower's sequences treated accordingly.

The alignment was then imported into Phylip, 100 replicates created and a distance-based matrix created using DNAdist. The neighbour joining method was then carried out and

the resulting tree replicated condensed using Consense (as in **Section 2.13.2**). Trees were visualised using Treeview. The resulting radial tree gives the traditional groupings, with a distinct division between slow and fast growers (Fig 4.1). Members of the *M. bovis/tuberculosis* group cluster separately as do those of the *M. avium/paratuberculosis* group. The internal relationships of the *M. bovis/tuberculosis* group are also represented in the tree, with *M. bovis, M. tuberculosis, M. ulcerans* and *M. leprae* all linked from a common ancestral tree branch.

4.4 *Mycobacterium* species diversity in soil

4.4.1 Primer design

Using the sequence alignment generated previously **(Section 4.3)**, a set of primers for use in the PCR were developed to specifically target the *Mycobacterium* genus. Sequences from species in other closely related genera (*Nocardia, Rhodococcus* and *Corynebacterium*) were included. Several candidates for forward and reverse primers were considered. Candidates were chosen from portions of the alignment where the sequence was identical in all the species from the mycobacteria (or 1 or 2 mismatches at most) and different from members of the other genera (7 or over mismatches) over a length of 25 to 35 residues. Potential primer sequences were also required to contain an even mix of A/T and C/G residues to produce a reasonable annealing temperature in the PCR. Finally forward and reverse primer candidates were chosen which would give a PCR product of sufficient length to accurately sequence and identify the product (over 400 bp). Primer candidates were imported into BlastN to determine their specificity, with primer set JSY16SF/R (shown in **Section 2.7.2**) finally chosen. Annealing temperatures for the PCR were devised using the formula $T = 2xA/T + 4xC/G$. The length of the expected PCR product gave an extension time of 1 min.

Fig 4.1. Neighbour-joined phylogenetic tree of relationships of 16S rRNA sequence data from the *Mycobacterium* genus. Sequences were obtained from Genbank and are named as either species names i.e. *M. bovis*, or as sequence names i.e. IWGMT90018, as presented in the database. Fast growers are shown in blue, slow growers in black, and the *M. tuberculosis/M. bovis* group in red. Bootstrap values of 100 replicate trees are shown.

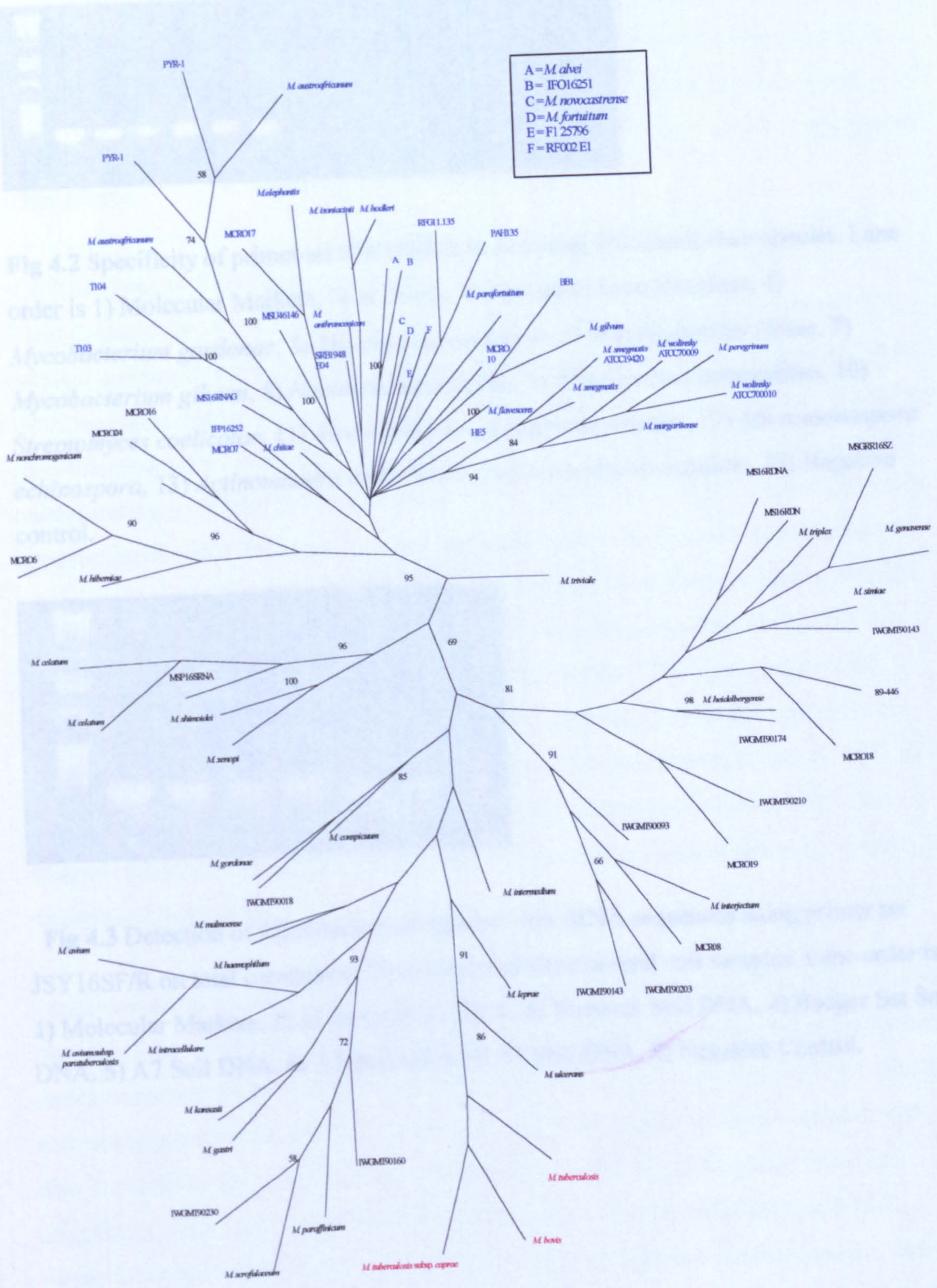

A=M. alvei
B= IFO16251
C=M. novocastrense
D=M. fortuitum
E=F1 25796
F=RF002 E1
PYR-1
M. austroafricanum
PYR-1
58
M. elephantis
M. iranicatti
M. hodleri
RFGI1.135
PAH135
74
MCRO17
M. austroafricanum
TI04
100
MSL146146
M. asiaticum
C
D
F
A
B
M. parafortuitum
BB1
TI03
100
SRB1948
ED4
100
E
MCRO
10
M. gilvum
M. smegmatis
ATCC19420
M. wolinsky
ATCC70009
M. peregrinum
MS16RNAG
100
M. smegmatis
M. wolinsky
ATCC700010
MCRO16
100
M. flavescens
MCRO24
IFP16252
HE5
M. margaritense
M. chitae
MCRO7
84
M. neoaurum
94
MSGRR16SZ
MS16RDNA
MS16RDN
M. triplex
M. genavense
90
96
MCRO6
M. hiberniae
95
M. triviale
M. simiae
IWGMT90143
96
69
M. celatum
MSP16SRNA
100
81
98
M. heidelbergense
89-446
M. celatum
M. shimoidei
IWGMT90174
M. xenopi
MCRO18
91
85
IWGMT90210
M. conspicuum
IWGMT90093
M. gordonae
66
MCRO19
M. intermedium
IWGMT90018
M. interjectum
M. malmoense
MCR08
M. avium
M. haemophilum
93
91
M. lepraemurium
IWGMT90143
IWGMT90203
M. intracellulare
72
86
M. ulcerans
M. avium subsp. paratuberculosis
M. kansasii
M. gastri
58
IWGMT90160
M. tuberculosis
IWGMT90230
M. paraffinicum
M. scrofulaceum
M. tuberculosis subsp. caprae
M. bovis
0.1

Fig 4.2 Specificity of primer set JSY16SF/R in detecting *Mycobacterium* species. Lane order is 1) Molecular Markers, 2) *M. bovis*, 3) *Mycobacterium fortuitum*, 4) *Mycobacterium gordonae*, 5) *Mycobacterium terrae*, 6) *Mycobacterium chitae*, 7) *Mycobacterium gilvum*, 8) *Nocardia brevicatena*, 9) *Rhodococcus coprophilus*, 10) *Streptomyces coelicolor*, 11) *Mycobacterium nonchromogenicum*, 12) *Micromonospora echinospora*, 13) *Actinomaudra malachitiea*, 14) *Arthrobacter oxydans*, 15) Negative control.

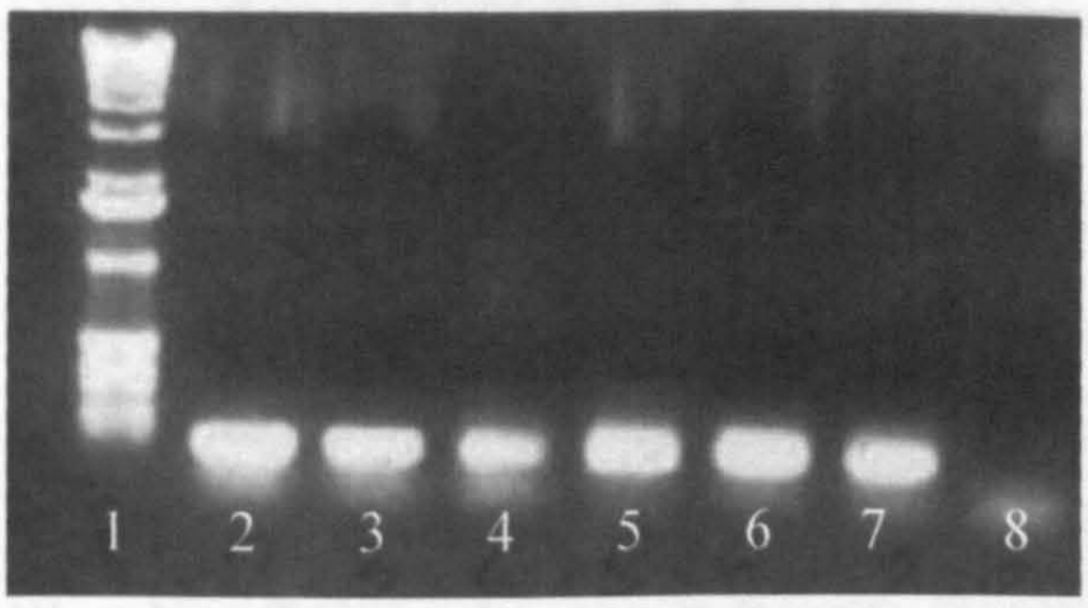


Fig 4.3 Detection of *Mycobacterium* species' 16S rRNA sequences using primer set JSY16SF/R on total community DNA extracted from several soil samples. Lane order is **1)** Molecular Markers. **2)** *M. bovis* BCG DNA. **3)** Warwick Soil DNA. **4)** Badger Set Soil DNA. **5**) A7 Soil DNA. **6)** A1 Soil DNA. **7)** A1 Soil DNA. **8)** Negative Control.

To determine the actual specificity of the primer pair, PCR was carried out on DNA extracted from selected organisms listed in Table 2.3.1. Products were visualised as in **Section 2.6.7,** the results of which can be seen in Fig 4.2. Primers were shown to be specific for members of the *Mycobacterium* genus with no product found in species from other genera.

4.4.2 Analysis of Total Community DNA

Total Community DNA was extracted from soil at the Irish farm sampling points BS1, A1, A7 (**Section 2.4**) and from Warwick soil (April 2000 sampling) as in **Section 2.6.3.** DNA was extracted from each of the ten cores from each site and subsequently pooled to give a representative community DNA sample from each sampling point. PCR was then carried out using JSY16SF/R and products visualised (Fig 4.3). As the resulting products appeared to be clean discrete bands they were cloned directly from the PCR reaction, negating the need to excise the bands from the gel. Each of the four soil DNA products was cloned and sequenced as in **Section 2.1.1.** Product from *M. bovis* BCG DNA was also cloned and sequenced to test efficacy of the procedure. This cloned sequenced matched 100% with *M. bovis* 16S rRNA encoding gene sequence when imported into Blast-N indicating the protocol was highly accurate. 50 clones were sequenced from each sampling site, Repeats were discarded and remaining sequences were imported into Blast-N to find the closest match in the Genbank database. These matches can be seen in Appendix 1, table A1.

Clone sequences were designated in the format G (for genus primers) followed by sample site and clone number i.e. badger set soil clone 1 would be GBS1.The sequences (plus the match sequences from Genbank) were aligned using ClustalW and imported into Bioedit then subsequently modified as in **Section 2.13.1.** Other sequences from Genbank were also imported to give a spread of known sequences from across the genus. Modified alignments were used in the construction of phylogenetic trees as in **Section 2.13.2.** Firstly DNAdist was used, this computes four different distances between species from nucleic acid sequences. The resulting matrix is then Neighbor-joined using Neighbor; this is a distance matrix method producing an unrooted tree without the assumption of a clock

All trees used *Nocardia asteroides* as an out-group, being a species from the closely related *Nocardia* genus. The resulting tree (Fig 4.4) shows two major groupings corresponding to the split between slow and fast growing mycobacteria, with the majority of clone sequences grouping in the fast growing section of the tree.

Only clones from the Warwick soil DNA library are represented in the slow growing section. The Irish soil clones from all three sites are spread across the fast growing section of the tree, with notable groupings in the *M. hiberniae* clade, the *M. murphy* clade and linked with an uncultured earthworm cast bacterium clone c211 sequence (Singleton *et al.*, unpublished data). This apparent bias towards sequences representing fast growing mycobacteria was investigated further (refer to **Section 4.5**).

To determine if the phylogenetic clusters had been represented fully, a second tree was created using the maximum-likelihood method (Fig 4.5). This model allows for unequal expected frequencies of the four nucleotides, for unequal rates of transitions and transversions, and for different (pre-specified) rates of change in different categories of sites, with the program inferring which sites have which rates. The major groupings present in the neighbour-joining tree were conserved, although there is a larger degree of separation on groupings. The position of the *M. hiberniae* clade is different in this tree, being grouped with the slow growers in the ML tree and with the fast growers in the NJ tree possibly due to the presence of the long helix in *M. hiberniae* 16S rRNA sequences and it being on the cusp of the slow/fast growing division. Again two Warwick soil clones are associated with *M. avium* and *M. avium* subsp. *paratuberculosis*. Clone groupings with *M. fallax, M. murphy* and *M. gordonae* are also conserved in the two trees. No clones grouped with the *M. bovis* clade.

Fig 4.4. Phylogenetic relationships of *Mycobacterium* species' 16S rRNA sequences from four soil clone libraries obtained using primer set JSY16SF/R, represented as a Neighbor-joining tree. Bootstrap values of 100 replicate trees are shown.

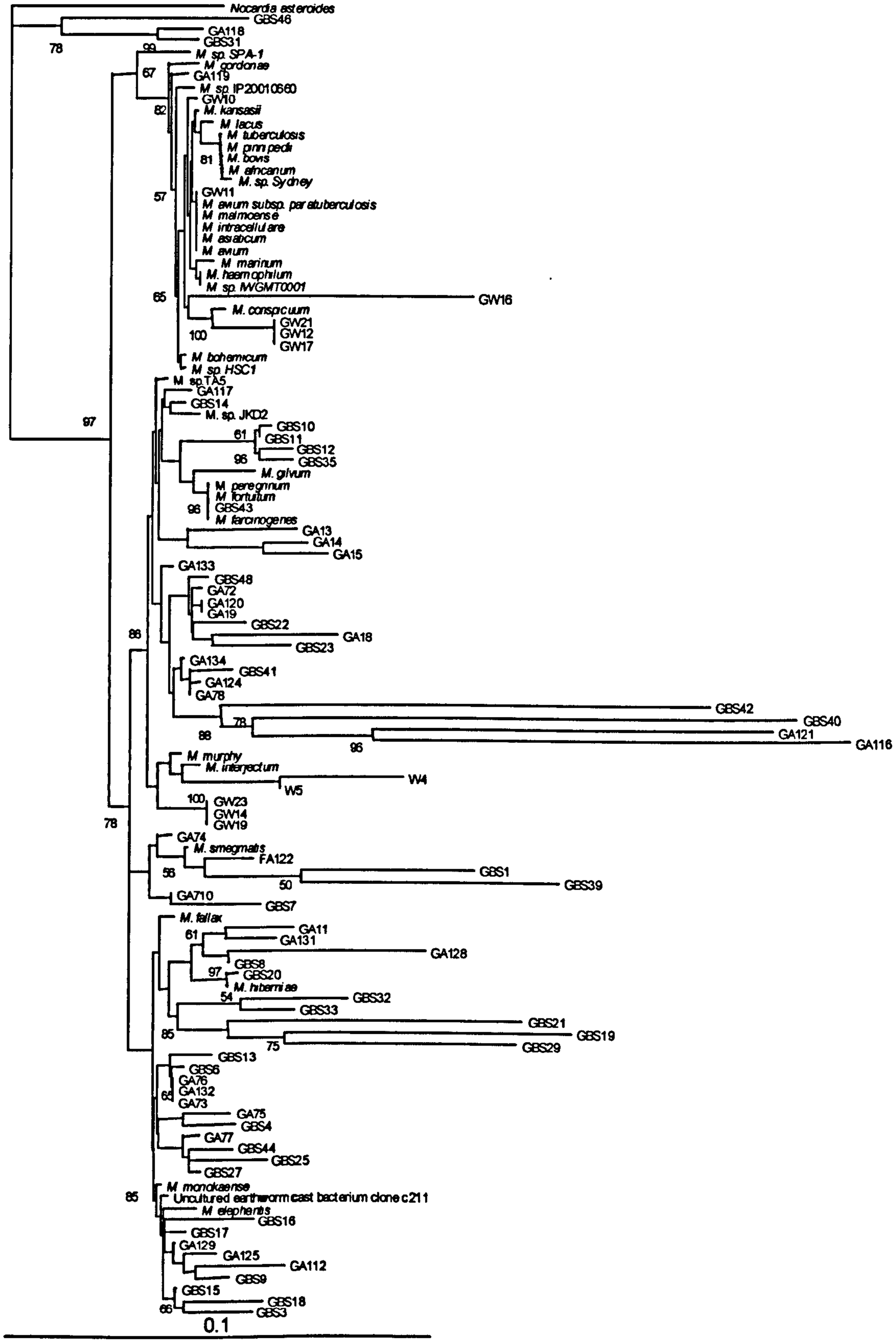
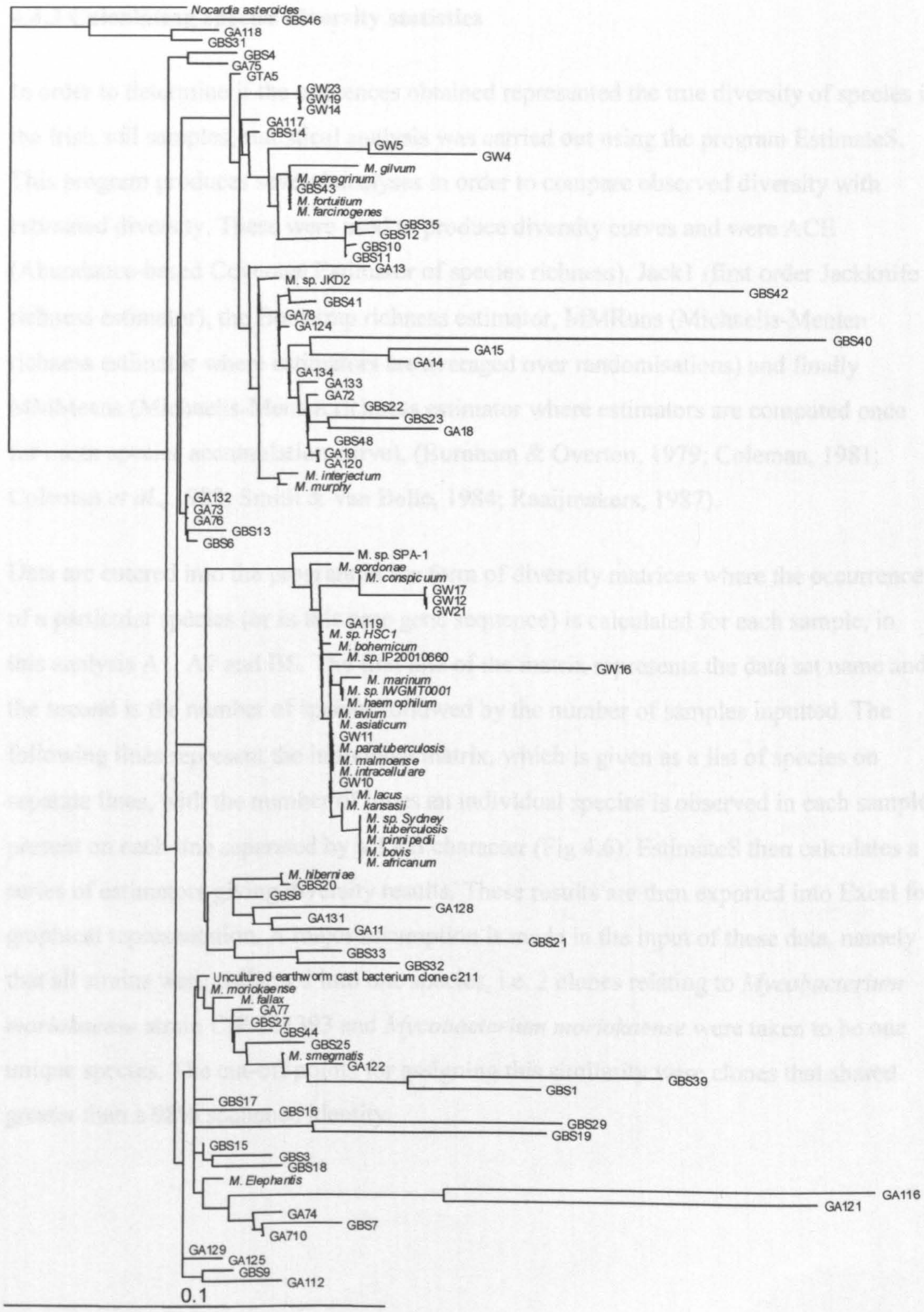

Nocardia asteroides
GBS46
GA118
GBS31
78
99
M. sp. SPA-1
M. gordonae
67
GA119
M. sp. IP20010660
GW10
82
M. kansasii
M. lacus
M. tuberculosis
M. pinnipedi
81
M. bovis
M. africanum
M. sp. Sydney
57
GW11
M. avium subsp. paratuberculosis
M. malmoense
M. intracellulare
M. asiaticum
M. avium
M. marinum
M. haemophilum
M. sp. IWGMT0001
65
GW16
M. conspicuum
GW21
100
GW12
GW17
M. bohemicum
M. sp. HSC1
M. sp.TA5
GA117
GBS14
M. sp. JKD2
97
GBS10
61
GBS11
GBS12
96
GBS35
M. gilvum
M. peregrinum
M. fortuitum
96
GBS43
M. farcinogenes
GA13
GA14
GA15
GA133
GBS48
GA72
GA120
GA19
GBS22
86
GA18
GBS23
GA134
GBS41
GA124
GA78
GBS42
78
GBS40
88
GA121
96
GA116
M. murphy
M. interjectum
W4
W5
100
GW23
GW14
78
GW19
GA74
M. smegmatis
FA122
56
GBS1
50
GBS39
GA710
GBS7
M. fallax
GA11
61
GA131
GA128
GBS8
97
GBS20
M. hibernia
54
GBS32
GBS33
GBS21
85
GBS19
75
GBS29
GBS13
GBS6
GA76
64
GA132
GA73
GA75
GBS4
GA77
GBS44
GBS25
GBS27
M. monokaense
85
Uncultured earthworm cast bacterium clone c211
M. elephantis
GBS16
GBS17
GA129
GA125
GA112
GBS9
GBS15
GBS18
66
GBS3
0.1

Fig 4.5. Phylogenetic relationships of *Mycobacterium* species' 16S rRNA sequences from four soil clone libraries obtained using primer set JSY16SF/R, represented as a maximum-likelihood derived tree.


Nocardia asteroides
GBS46
GA118
GBS31
GBS4
GA75
GTA5
GW23
GW19
GW14
GA117
GBS14
GW5
GW4
M. gilvum
M. peregrinum
GBS43
M. fortuitium
M. farcinogenes
GBS35
GBS12
GBS10
GBS11
GA13
M. sp. JKD2
GBS42
GBS41
GA78
GA124
GBS40
GA15
GA14
GA134
GA133
GA72
GBS22
GBS23
GA18
GBS48
GA19
GA120
M. interjectum
M. murphy
GA132
GA73
GA76
GBS13
GBS6
M. sp. SPA-1
M. gordonae
M. conspicuum
GW17
GW12
GW21
GA119
M. sp. HSC1
M. bohemicum
M. sp. IP20010660
GW16
M. marinum
M. sp. IWGMT0001
M. haemophilum
M. avium
M. asiaticum
GW11
M. paratuberculosis
M. malmoense
M. intracellulare
GW10
M. lacus
M. kansasii
M. sp. Sydney
M. tuberculosis
M. pinnipedii
M. bovis
M. africanum
M. hiberniae
GBS20
GBS8
GA128
GA131
GA11
GBS21
GBS33
GBS32
Uncultured earthworm cast bacterium clone c211
M. moriokaense
M. fallax
GA77
GBS27
GBS44
GBS25
M. smegmatis
GA122
GBS39
GBS1
GBS17
GBS16
GBS29
GBS19
GBS15
GBS3
GBS18
M. Elephantis
GA116
GA121
GA74
GBS7
GA710
GA129
GA125
GBS9
GA112
0.1

4.4.3 Calculating species diversity statistics

In order to determine if the sequences obtained represented the true diversity of species in the Irish soil samples, statistical analysis was carried out using the program EstimateS. This program produces several analyses in order to compare observed diversity with estimated diversity. These were used to produce diversity curves and were ACE (Abundance-based Coverage Estimator of species richness), Jack1 (first order Jackknife richness estimator), the Bootstrap richness estimator, MMRuns (Michaelis-Menten richness estimator where estimators are averaged over randomisations) and finally MMMeans (Michaelis-Menten richness estimator where estimators are computed once for mean species accumulation curve), (Burnham & Overton, 1979; Coleman, 1981; Coleman *et al.*, 1982; Smith & van Belle, 1984; Raaijmakers, 1987).

Data are entered into the program in the form of diversity matrices where the occurrence of a particular species (or in this case gene sequence) is calculated for each sample, in this analysis A1, A7 and BS. The first line of the matrix represents the data set name and the second is the number of species followed by the number of samples inputted. The following lines represent the individual matrix, which is given as a list of species on separate lines, with the number of times an individual species is observed in each sample present on each line separated by a TAB character (Fig 4.6). EstimateS then calculates a series of estimators giving diversity results. These results are then exported into Excel for graphical representation. A major assumption is made in the input of these data, namely that all strains were collapsed into one species, i.e. 2 clones relating to *Mycobacterium moriokaense* strain CIP 105393 and *Mycobacterium moriokaense* were taken to be one unique species. The cut-off points for assigning this similarity were clones that shared greater than a 98% sequence identity.

```
Fast
35      3
1       0       1
1       0       0
4       0       2
2       0       1
1       0       0
1       0       2
1       0       0
2       0       1
1       0       1
1       0       1…
```

Fig 4.6. Input matrix derived for use in EstimateS to determine diversity and shared statistics of soil clone libraries.

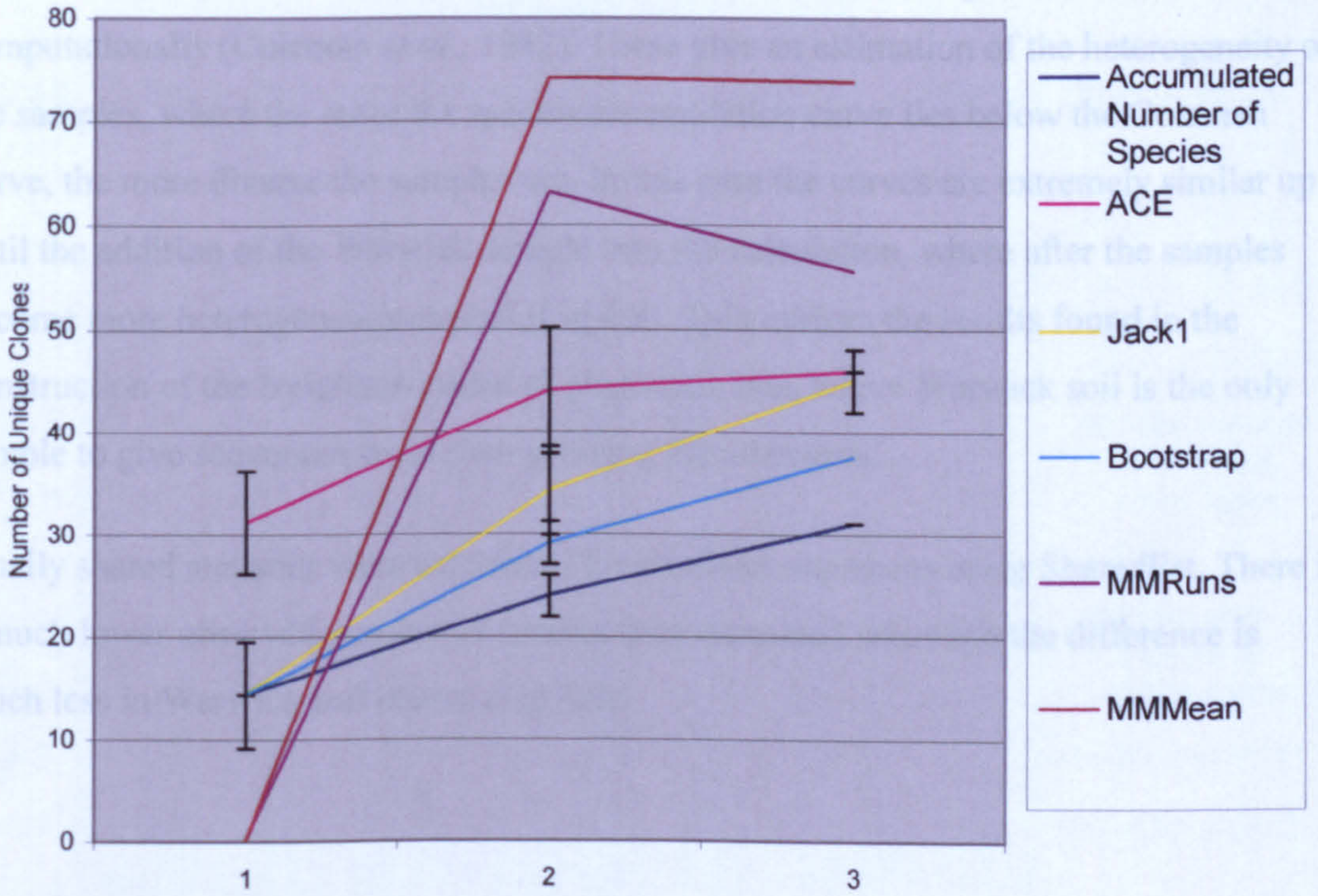


Fig 4.7. Derived diversity statistics for three Irish soil clone libraries (BS1, A1 and A7), created using EstimateS.

In Fig 4.7 the observed number of species is much lower for each sample than the estimated diversity in the Irish soil samples, for Jack1, ACE, and Bootstrap over the three samples. Bootstrap gives an estimated diversity of approximately six species more than observed, Jack1 and ACE give fourteen more species than observed. Standard deviations of the estimators were calculated from values of 50 randomisations. The MMmean curve gives a richness estimator much higher than the other estimators, due to MMmean calculated species richness in all samples together, rather than in single samples as previously. The MMrun results follow this trend but at approximately 20 species. MMruns are known to highly over-estimate richness when a species rich sample is added to the calculation (as BS is), which skews the resulting curve.

Coleman curves were calculated for all samples including Warwick clones to determine the overall efficacy of species identification (Coleman, 1981, Coleman *et al.*, 1982). These curves replace rarefaction curves, as they are orders of magnitude more efficient computationally (Coleman *et al.*, 1982). These give an estimation of the heterogeneity of the samples, where the more the species accumulation curve lies below the Coleman curve, the more diverse the samples are. In this case the curves are extremely similar up until the addition of the Warwick sample into the calculation, where after the samples become more heterogeneous overall (Fig 4.8). This mirrors the results found in the construction of the Neighbor-joined phylogenetic tree, where Warwick soil is the only sample to give sequences from slow growing mycobacteria.

Finally shared statistics were calculated for all clone sequences using SharedEst. There is a much lower observed number of species than estimated, although the difference is much less in Warwick soil clones (Fig 4.9).

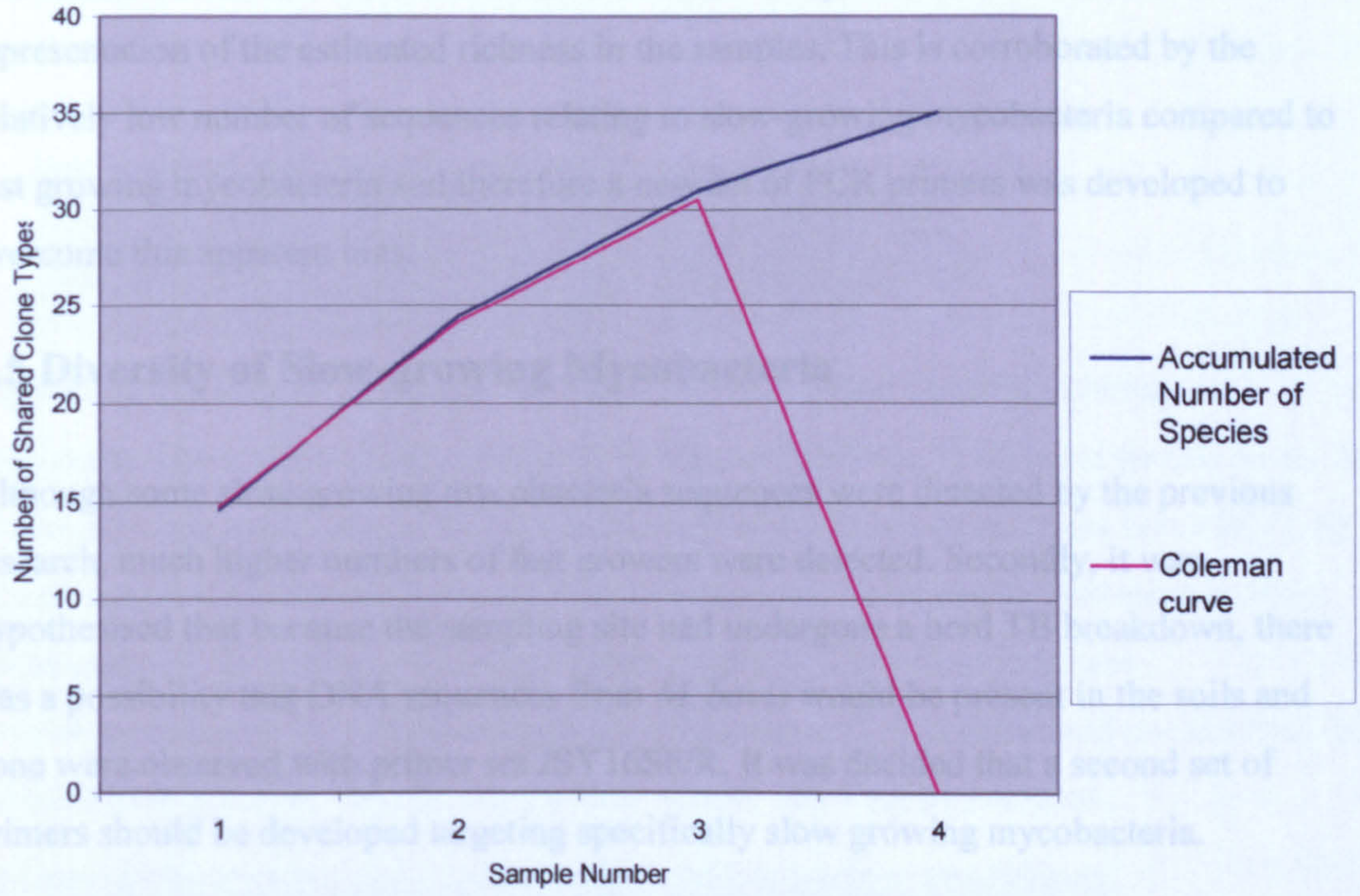


Fig 4.8. Coleman curve calculated from diversity statistics of four soil clone libraries. 1 = A1, 2 = A7, 3 = BS and 4 = W.

Fig 4.9. Shared statistics for four soil clone libraries calculated using EstimateS.

The results overall show that the number of observed species cannot be taken as a true representation of the estimated richness in the samples. This is corroborated by the relatively low number of sequences relating to slow-growing mycobacteria compared to fast growing mycobacteria and therefore a new set of PCR primers was developed to overcome this apparent bias.

4.5 Diversity of Slow-growing Mycobacteria

Although some slow-growing mycobacteria sequences were detected by the previous research, much higher numbers of fast growers were detected. Secondly, it was hypothesised that because the sampling site had undergone a herd TB breakdown, there was a possibility that DNA sequences from *M. bovis* would be present in the soils and none were observed with primer set JSY16SF/R. It was decided that a second set of primers should be developed targeting specifically slow growing mycobacteria.

4.5.1 Primer design

The original alignment was modified, by removing all the fast grower sequences and the forward primer was based around the long helix at position 452, again with maximum similarity to *Mycobacterium* sequences, and minimal similarity to sequences from other genera. Primers were chosen by the same criteria as before. JSY16SslowF/R were finally chosen (Table 2.7.2).

4.5.2 Analysing slow-grower 16S rRNA sequences in total community DNA

Again the specificity of these primers was tested in a PCR using DNA extracted form the species listed in **Section 2.3.1**. The results from this PCR can be seen in Fig 4.10. PCR was then carried out on the same, pooled community DNA samples as before, and the products visualised, the results can be seen in Fig 4.11. PCR products were cloned and sequenced as in **Section 2.12.**

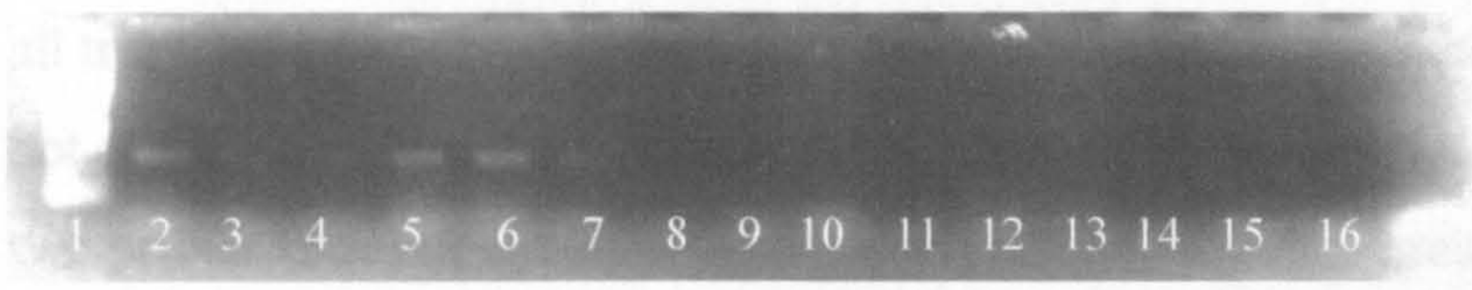


Fig 4.10 Specificity of primer set JSY16SslowF/R in detecting slow growing *Mycobacterium* species. Lane order is 1) Molecular Markers, 2) *Mycobacterium bovis*, 3) *Mycobacterium tuberculosis*, 4) *Mycobacterium bovis* 5) *Mycobacterium bovis* BCG, 6) *Mycobacterium gordonae*, 7) *Mycobacterium marinum* 8) *Nocardia brevicatena*, 9) *Rhodococcus coprophilus*, 10) *Mycobacterium chitae*, 11) *Mycobacterium nonchromogenicum*, 12) *Micromonospora echinospora*, 13) *Actinomaudra malachite*, 14) *Arthrobacter oxydans*, 15) *Mycobacterium gilvum,* 16) Negative control.

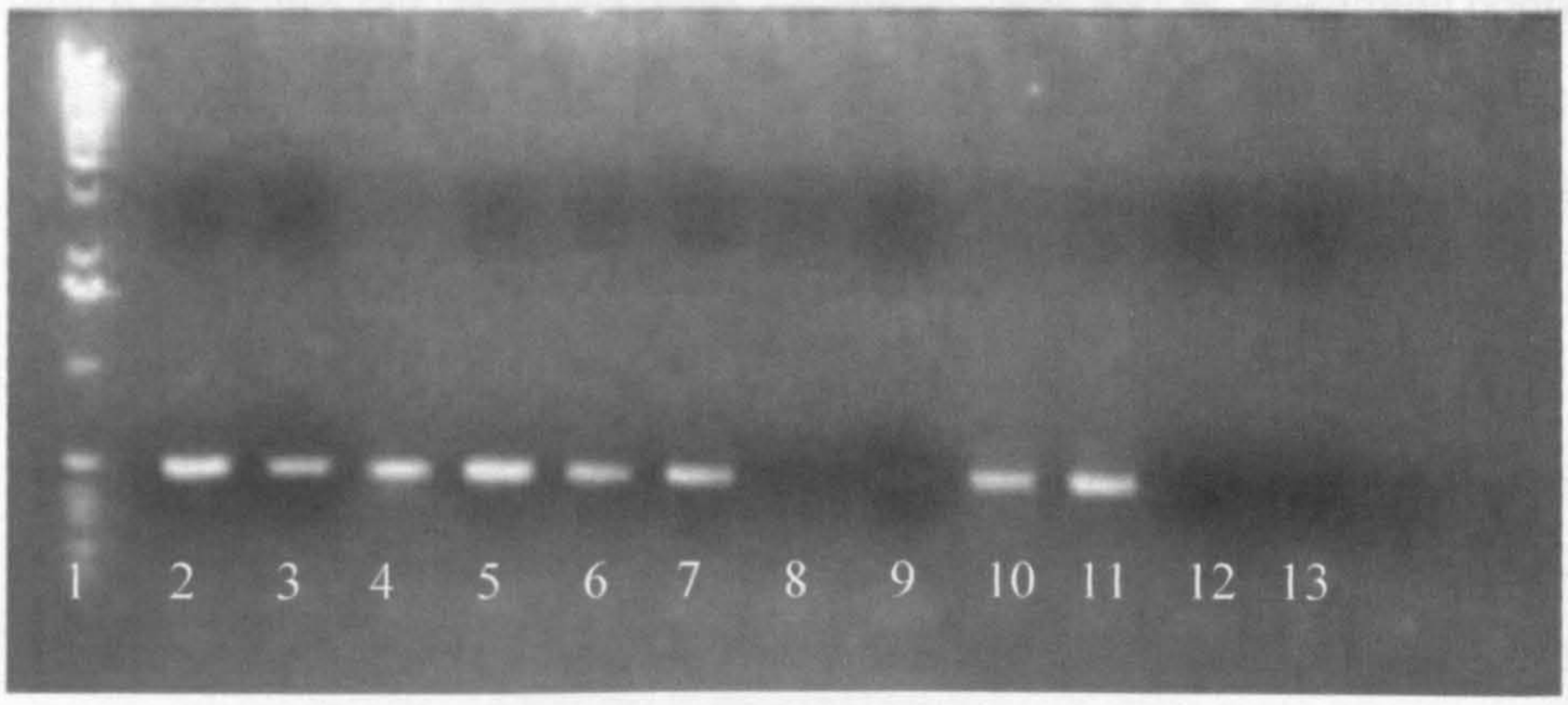


Fig 4.11 Detection of *Mycobacterium* species' 16S rRNA sequences using primer set JSY16SF/R on total community DNA extracted from several soil samples. Lane order is **1)**, Molecular weight markers, **2)** *M. bovis* DNA, **3)** *M. bovis* BCG DNA **4, 5)** Badger set soil DNA, **6, 7)** DNA from soil at the University of Warwick, **8, 9)** A7 soil, **10, 11)** A1 Soil, **12, 13)** Negative control.

Fifty sequences were obtained from each sample and duplicate sequences discarded. Sequences were then used to construct neighbor-joined and maximum-likelihood trees as in **Section 2.13.** Where possible the same known sequences from Genbank were used in all trees, however in some cases this was not possible as the submitted sequences were not of sufficient length to cover both the areas of the 16S rRNA gene obtained using the two primer sets. For example the *M. moriokaense* sequence present in Genbank stops approximately 100bp into the product obtained using JSY16SslowF/R, but the area of the gene covered by JSY16SF/R is present.

Using this primer set no sequences from fast-growing mycobacteria were detected with only type strain sequences represented in the neighbour-joining tree (Fig 4.12). There are a high number of sequences, which share clades and come from different samples, e.g. the *M. gordonae* clade has clone sequences from A1 and W samples. Also the clade which separates out, from between *M. interjectum* and *M. gordonae* contains clones from A7, BS and W samples. The clones grouping with the *M. avium* clade are again present and are all from Warwick soil. Finally the first occurrence of sequences grouping with *M. bovis* were seen from both badger set soils and A1 soils.

To verify clade and group positions a maximum-likelihood derived tree was generated and as before the groupings are maintained, but a better separation is observed (Fig 4.13). The sequences grouping with *M. bovis* are again present at the same location as are the *M. gordonae* group and the clade between *M. gordonae* and *M. bovis.* The three Warwick clones which grouped with *M. avium* using NJ (SW1, SW2 and SW5) are more divergent from the *M. avium* group using ML, although the branch originates in the group suggesting an evolutionary step from *M. avium* in these sequences.

Fig 4.12. Diversity of slow-growing *Mycobacterium* spp. 16S rRNA sequences from four soil clone libraries, represented as a neighbor-joining bootstrapped phylogenetic tree.

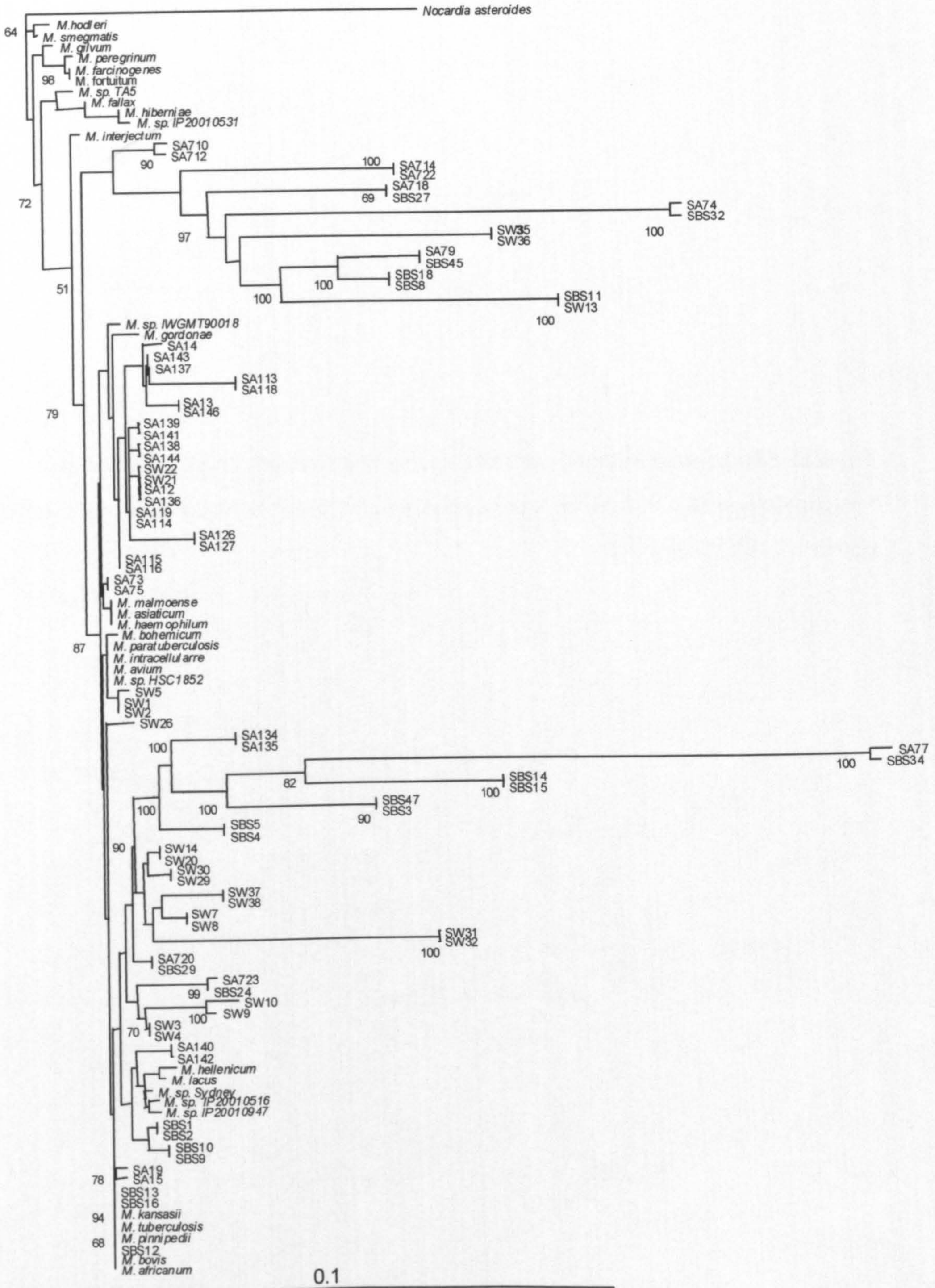
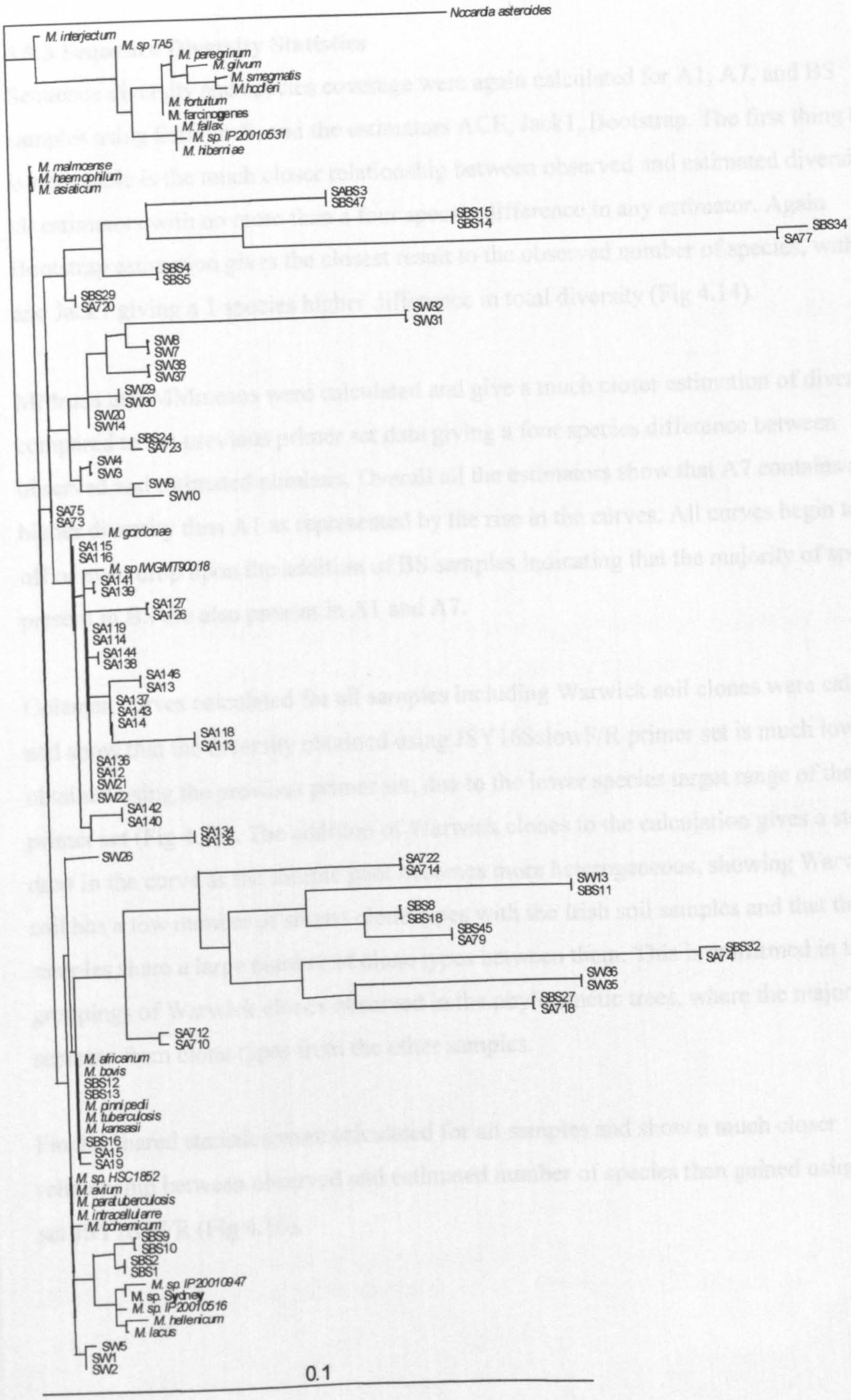

Nocardia asteroides
M.hodleri
M. smegmatis
M. gilvum
M. peregrinum
M. farcinogenes
M. fortuitum
M. sp. TA5
M. fallax
M. hiberniae
M. sp. IP20010531
M. interjectum
SA710
SA712
SA714
SA722
SA718
SBS27
SA74
SBS32
SW35
SW36
SA79
SBS45
SBS18
SBS8
SBS11
SW13
M. sp. IWGMT90018
M. gordonae
SA14
SA143
SA137
SA113
SA118
SA13
SA146
SA139
SA141
SA138
SA144
SW22
SW21
SA12
SA136
SA119
SA114
SA126
SA127
SA115
SA116
SA73
SA75
M. malmoense
M. asiaticum
M. haemophilum
M. bohemicum
M. paratuberculosis
M. intracellulare
M. avium
M. sp. HSC1852
SW5
SW1
SW2
SW26
SA134
SA135
SA77
SBS34
SBS14
SBS15
SBS47
SBS3
SBS5
SBS4
SW14
SW20
SW30
SW29
SW37
SW38
SW7
SW8
SW31
SW32
SA720
SBS29
SA723
SBS24
SW10
SW9
SW3
SW4
SA140
SA142
M. hellenicum
M. lacus
M. sp. Sydney
M. sp. IP20010516
M. sp. IP20010947
SBS1
SBS2
SBS10
SBS9
SA19
SA15
SBS13
SBS16
M. kansasii
M. tuberculosis
M. pinnipedii
SBS12
M. bovis
M. africanum
0.1

Fig 4.13. Maximum-likelihood derived tree showing diversity of slow-growing *Mycobacterium* spp. 16S rRNA sequences among four soil clone libraries obtained using primer set JSY16SslowF/R


Nocardia asteroides
M. interjectum
M. sp TA5
M. peregrinum
M. gilvum
M. smegmatis
M.hodleri
M. fortuitum
M. farcinogenes
M. fallax
M. sp. IP20010531
M. hiberniae
M. malmoense
M. haemophilum
M. asiaticum
SABS3
SBS47
SBS15
SBS14
SBS34
SA77
SBS4
SBS5
SBS29
SA720
SW32
SW31
SW8
SW7
SW38
SW37
SW29
SW30
SW20
SW14
SBS24
SA723
SW4
SW3
SW9
SW10
SA75
SA73
M. gordonae
SA115
SA116
M. sp IWGMT90018
SA141
SA139
SA127
SA126
SA119
SA114
SA144
SA138
SA146
SA13
SA137
SA143
SA14
SA118
SA113
SA136
SA12
SW21
SW22
SA142
SA140
SA134
SA135
SW26
SA722
SA714
SW13
SBS11
SBS8
SBS18
SBS45
SA79
SBS32
SA74
SW36
SW35
SBS27
SA718
SA712
SA710
M. africanum
M. bovis
SBS12
SBS13
M. pinnipedi
M. tuberculosis
M. kansasii
SBS16
SA15
SA19
M. sp. HSC1852
M. avium
M. paratuberculosis
M. intracellularre
M. bohemicum
SBS9
SBS10
SBS2
SBS1
M. sp. IP20010947
M. sp. Sydney
M. sp. IP20010516
M. hellericum
M. lacus
SW5
SW1
SW2
0.1

4.5.3 Sequence Diversity Statistics

Sequence diversity and species coverage were again calculated for A1, A7, and BS samples using EstimateS, and the estimators ACE, Jack1, Bootstrap. The first thing that is noticeable is the much closer relationship between observed and estimated diversity for all estimators with no more than a four species difference in any estimator. Again Bootstrap estimation gives the closest result to the observed number of species, with ACE and Jack1 giving a 1 species higher difference in total diversity (Fig 4.14).

MMruns and MMmeans were calculated and give a much closer estimation of diversity compared to the previous primer set data giving a four species difference between observed and estimated numbers. Overall all the estimators show that A7 contains a higher diversity than A1 as represented by the rise in the curves. All curves begin to level off or even drop upon the addition of BS samples indicating that the majority of species present in BS are also present in A1 and A7.

Coleman curves calculated for all samples including Warwick soil clones were calculated and show that the diversity obtained using JSY16SslowF/R primer set is much lower than obtained using the previous primer set, due to the lower species target range of the new primer set (Fig 4.15). The addition of Warwick clones to the calculation gives a steep drop in the curve as the sample pool becomes more heterogeneous, showing Warwick soil has a low number of shared clone types with the Irish soil samples and that the Irish samples share a large number of clone types between them. This is confirmed in the groupings of Warwick clones observed in the phylogenetic trees, where the majority are separate from clone types from the other samples.

Finally shared statistics were calculated for all samples and show a much closer relationship between observed and estimated number of species than gained using primer set JSY16SF/R (Fig 4.16).

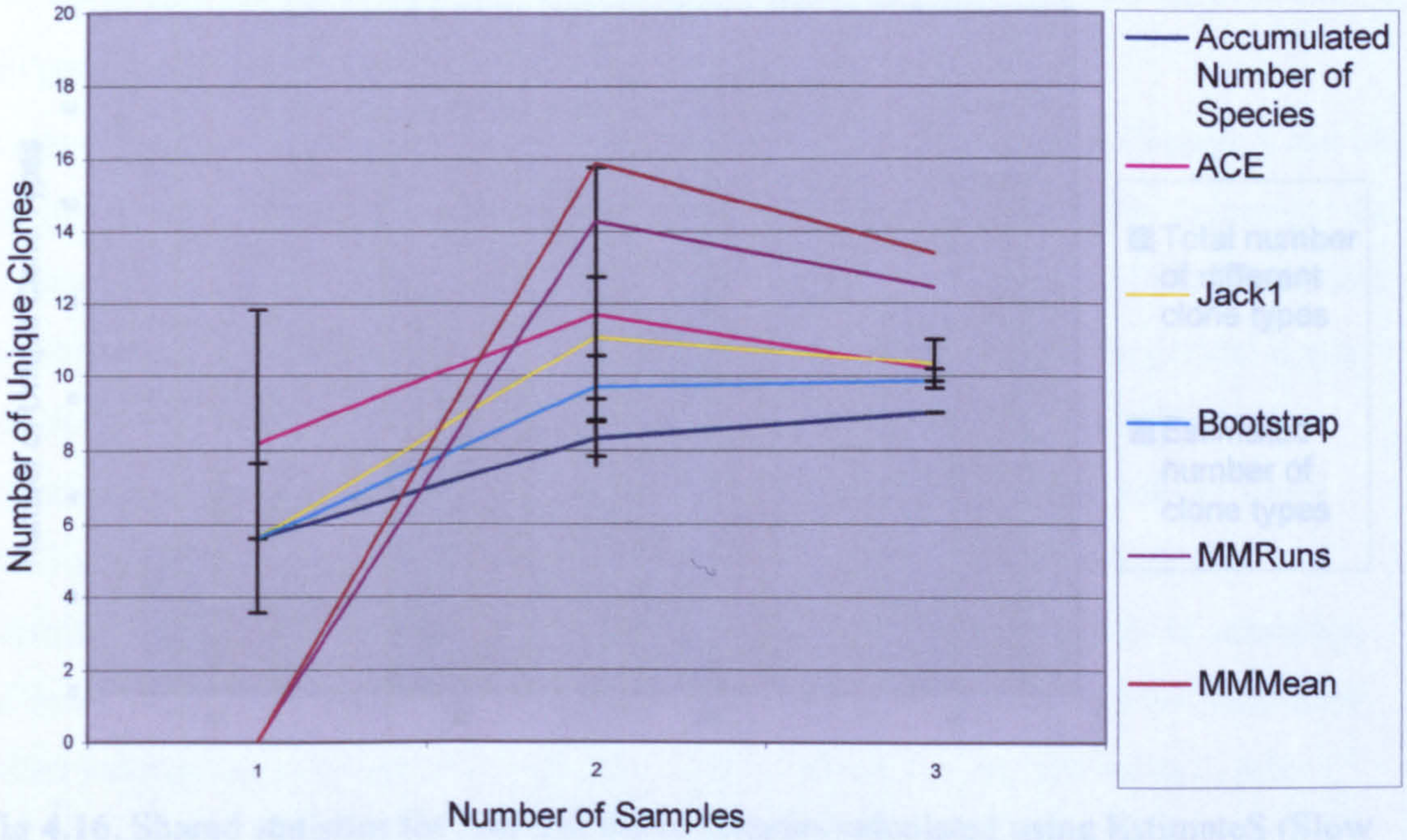


Fig 4.14. Derived diversity statistics for slow growing *Mycobacterium* species from three Irish soil clone libraries (BS1, A1 and A7), created using EstimateS.

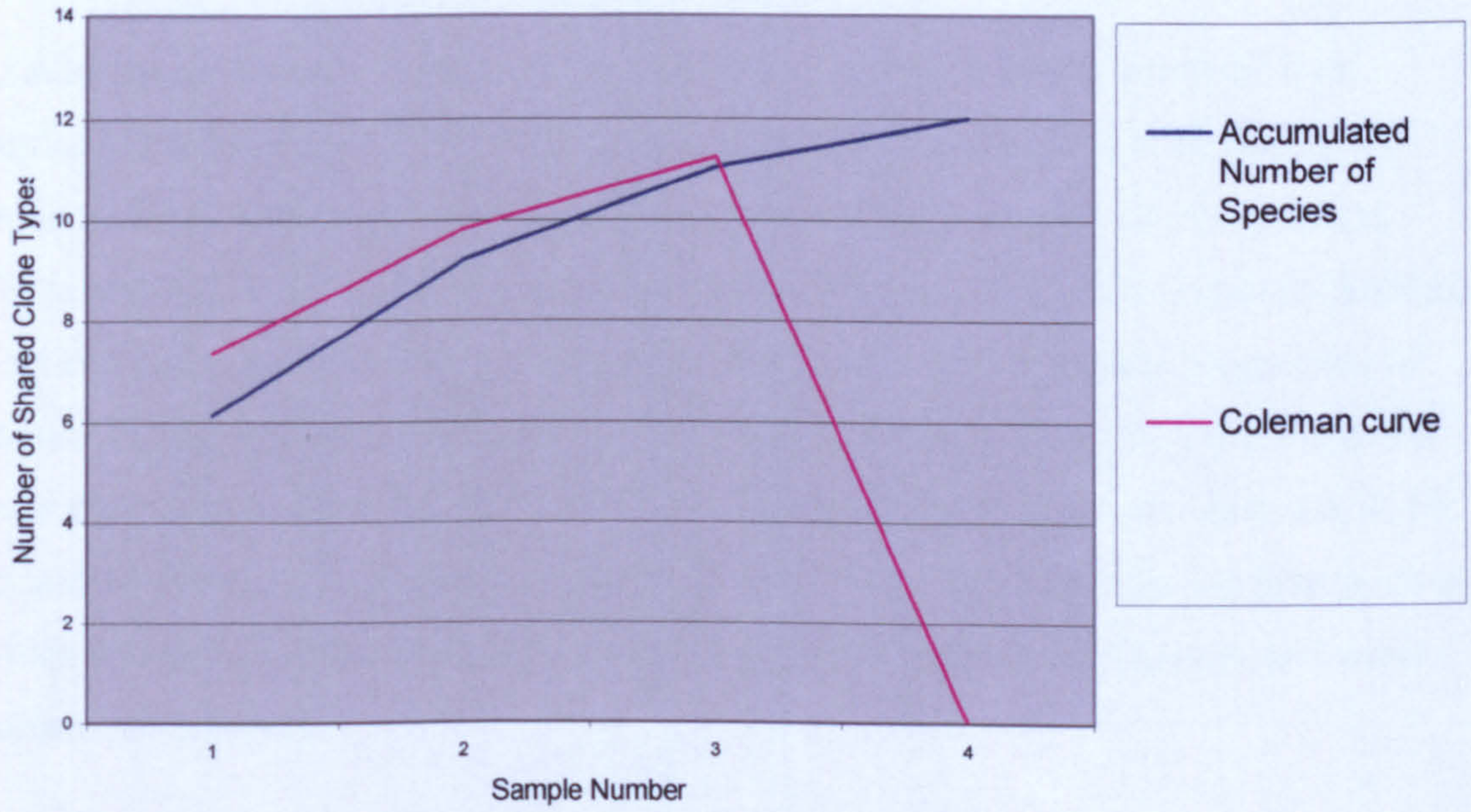


Fig 4.15. Coleman curve calculated from diversity statistics of four soil clone libraries (Slow growing *Mycobacterium* species). 1 = A1, 2 = A7, 3 = BS and 4 = W.

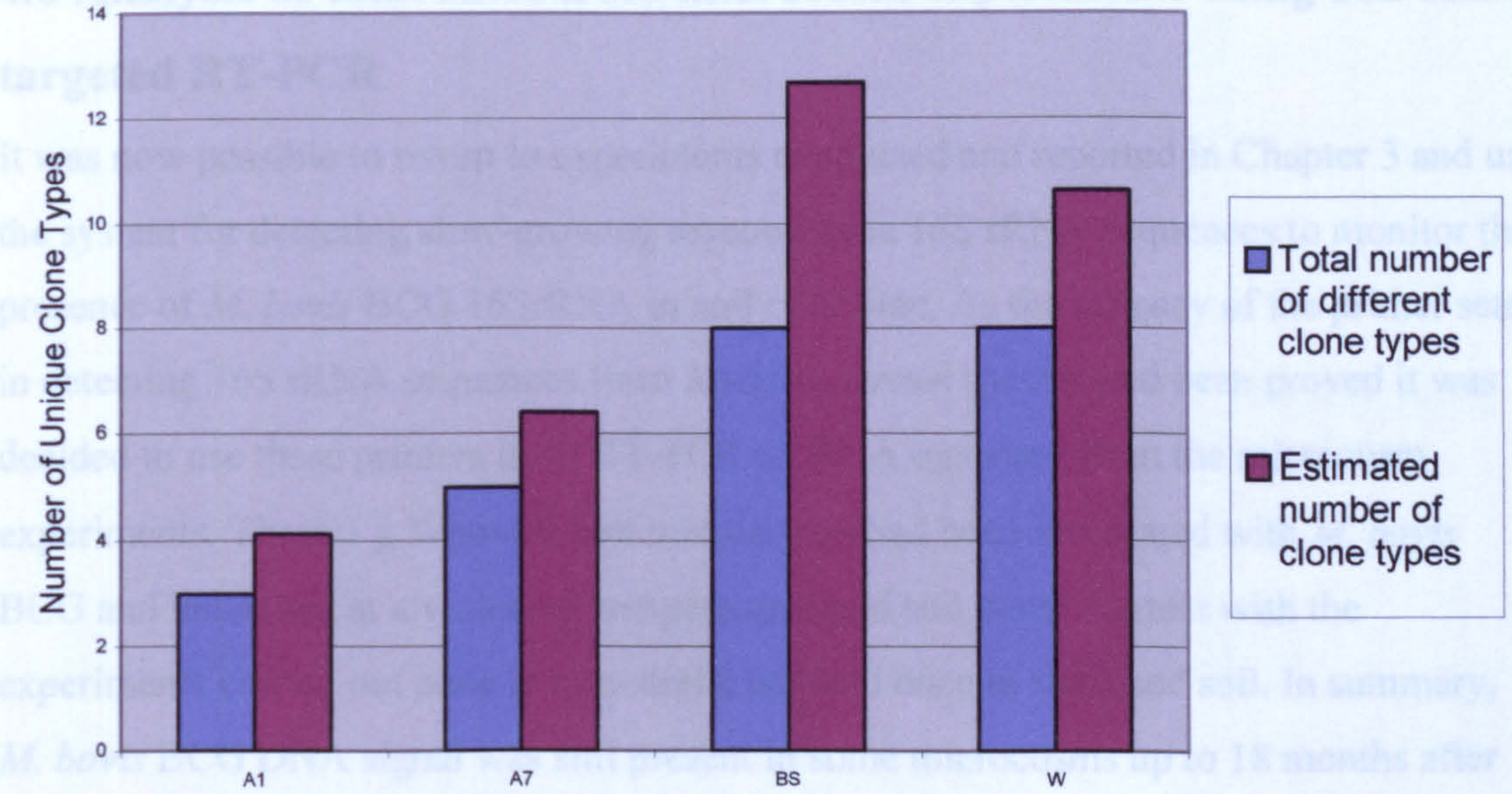


Fig 4.16. Shared statistics for four soil clone libraries calculated using EstimateS (Slow growing *Mycobacterium* species).

4.6 Analysis of total RNA from microcosm experiments using 16S rRNA targeted RT-PCR

It was now possible to return to experiments conducted and reported in Chapter 3 and use the system for detecting slow-growing mycobacteria 16S rRNA sequences to monitor the presence of *M. bovis* BCG 16SrRNA in soil over time. As the efficacy of the primer sets in detecting 16S rRNA sequences from *Mycobacterium* species had been proved it was decided to use these primers in an RT-PCR on RNA extracted from the microcosm experiments. These 1 g Warwick soil microcosms had been inoculated with *M. bovis* BCG and incubated at a variety of temperatures and soil water content with the experiments carried out once in non-sterile soil and once in sterilised soil. In summary, *M. bovis* BCG DNA signal was still present in some microcosms up to 18 months after initial inoculation, whereas mRNA signal was not detected in any microcosms after 1 day. Taking both these facts into account the detection of the presence, or lack of, 16S rRNA signal would determine the nature of the *M. bovis* BCG cells in the microcosms i.e. dormant, dead or viable (Desjardin *et al.*, 1999).

At selected time points throughout the experiments, total RNA was extracted from duplicate microcosms and RT-PCR carried out using the JSY16SslowF/R primer set, clone libraries were created and selected clones underwent plasmid DNA extraction. These plasmids were then partially sequenced to cover the 16S cDNA sequence (**Sections 2.11** and **2.22**). As sequences from *M. bovis* BCG only were of interest, sequencing of clones was stopped once a relevant sequence had been discovered. RNA was extracted from the microcosms at 1, 3, 5, 10, 20, 30 and 60 days after inoculation, then at 4, 6, 10, 15 and 18 months. Results are presented in Fig 4.17 and Fig 4.18 as the last time point at which relevant 16S rRNA sequences were detected for both the temperature and water content experiments.

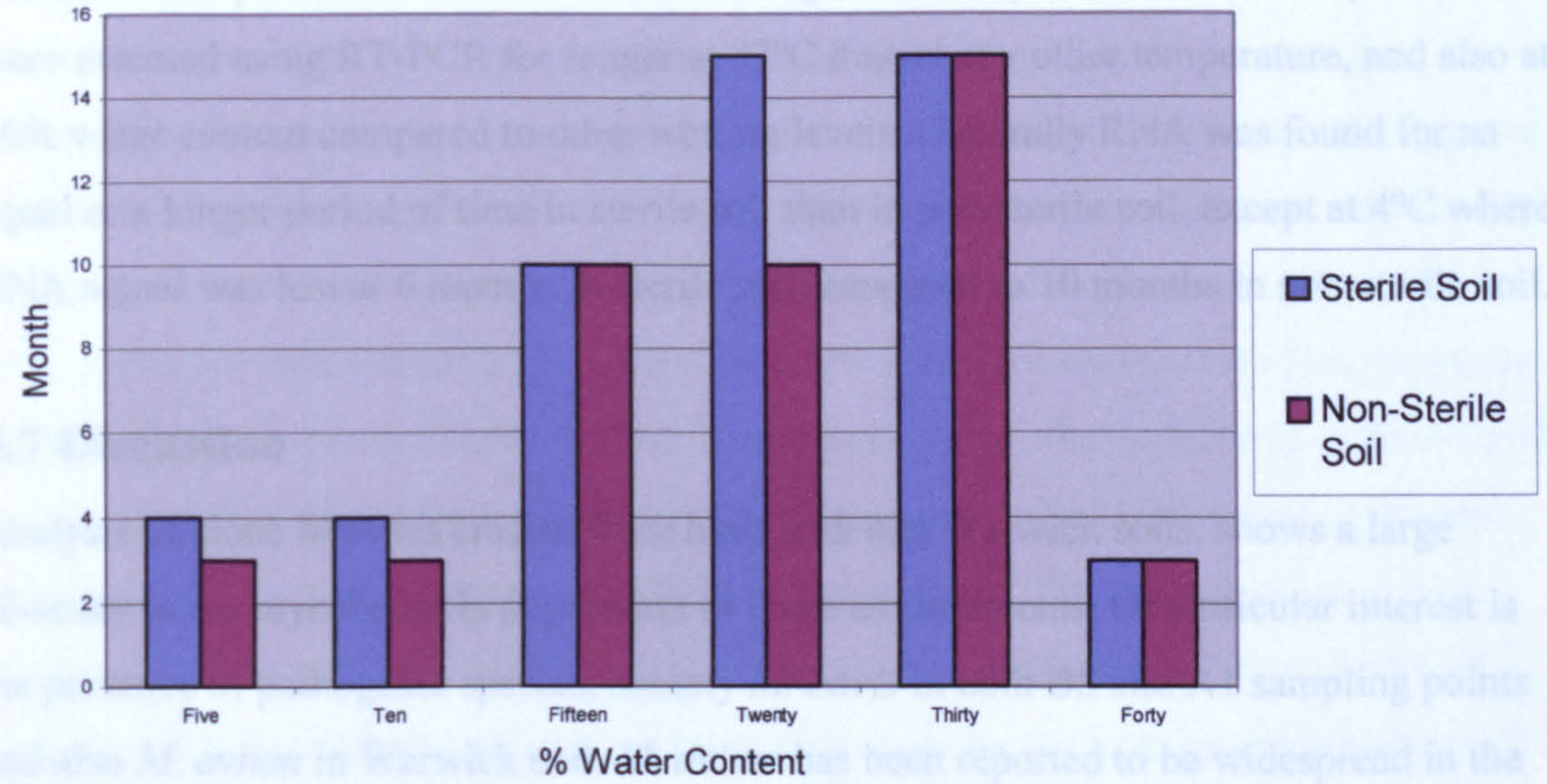


Fig 4.17. Survival of *M. bovis* BCG as represented by 16S rRNA detected by RT-PCR. Values represent the time in months at which RNA signal could be last detected in microcosms held at different wetting levels.

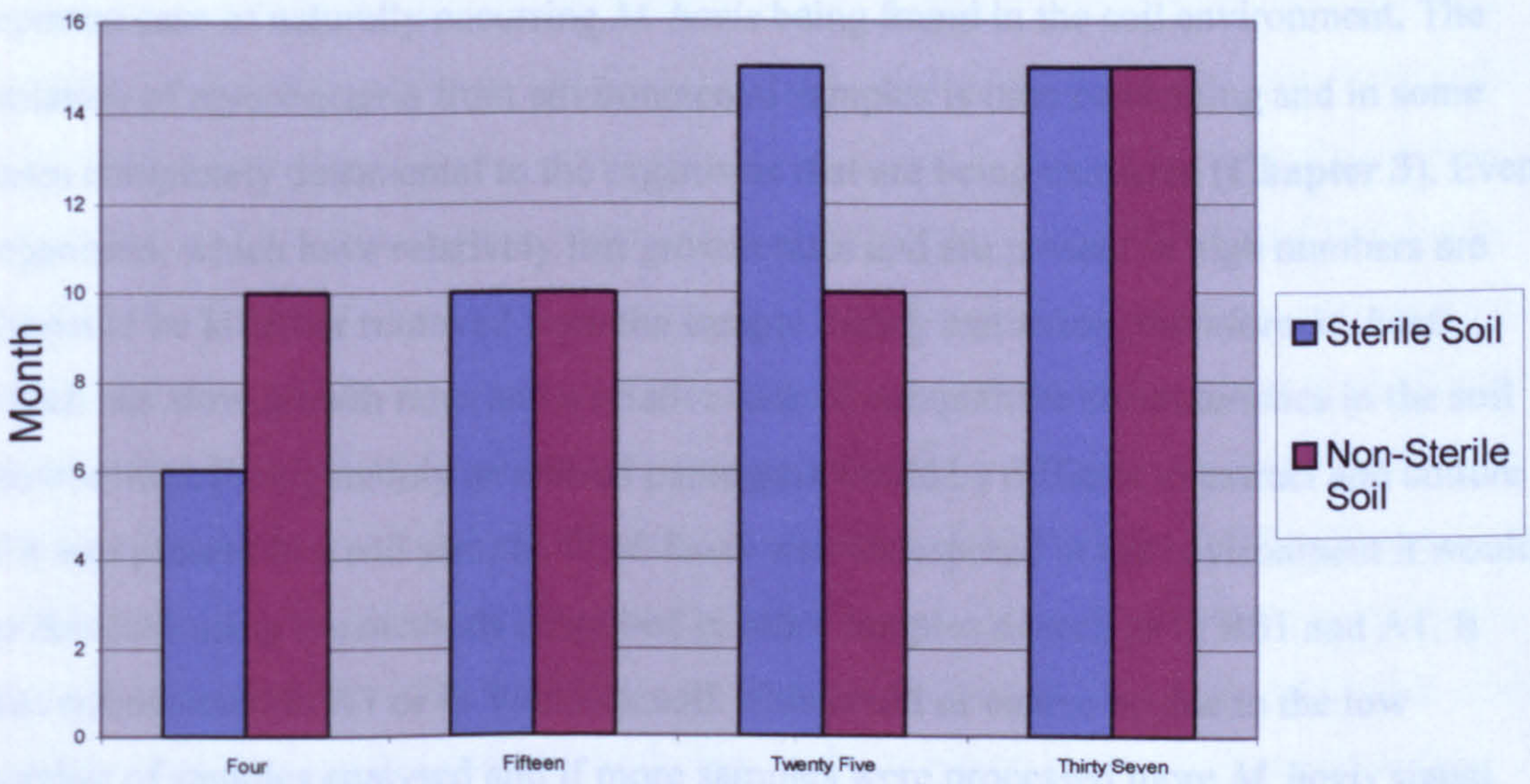


Fig 4.18. Survival of *M. bovis* BCG, represented by 16S rRNA detected by RT-PCR. Values represent the time in months at which RNA signal could last be detected in microcosms held at different temperatures.

The RNA analysis confirms data obtained using DNA analysis. 16S rRNA sequences were detected using RT-PCR for longer at 37°C than at any other temperature, and also at 30% water content compared to other wetting levels. Generally RNA was found for an equal or a longer period of time in sterile soil than in non-sterile soil, except at 4°C where RNA signal was lost at 6 months in sterile soil compared to 10 months in non-sterile soil.

4.7 Discussion

Analysis of clone libraries created from both Irish and Warwick soils, shows a large diversity in the mycobacteria population of these environments. Of particular interest is the presence of pathogenic species, namely *M. bovis* in both BS and A1 sampling points and also *M. avium* in Warwick soil. *M. avium* has been reported to be widespread in the environment, being isolated previously from soil, water and dust particles (Prosser, 1989; Limia *et al.*, 2001; Fischer *et al.*, 2003) and similar sequences were found in Warwick soil, with NJ giving a closer relationship between the *M. avium* type-strain sequence and the clone sequence than ML analysis. The detection of *M. bovis* however, is the first reported case of naturally occurring *M. bovis* being found in the soil environment. The isolation of mycobacteria from environmental samples is time consuming and in some cases completely detrimental to the organisms that are being extracted (**Chapter 3**). Even organisms, which have relatively fast growth rates and are present in high numbers are shown to be killed or removed from the sample during extraction, therefore *M. bovis* which has slow growth rates and a relative lack of competitive characteristics in the soil environment (being mainly an animal pathogen) would be difficult to extract and culture if it was present in a soil sample. If *M. bovis* was widespread in the environment it would be detected using the methods described in other samples as well as in BS1 and A1. It was not detected in A7 or in Warwick soil. This could of course be due to the low number of samples analysed and if more samples were processed more *M. bovis* signal could be detected. However from studies presented in Chapter 5, where presence is localised, and the data in this chapter it is concluded that *M. bovis* is only found in soils which have been in direct prolonged contact with infected animals, and the route of transmission to the soil is through infected secretions from these animals. Comparison may be made in relation to other species detected. Certain species (or more accurately

16S rRNA clone types) are present across the three Irish soil samples, but also in Warwick soil i.e. the clone types grouping with *M. farcinogenes* so it appears that differing environments share some species groups if *M. bovis* shares this apparent spread, then it could indeed be present in different geographical locations. However, as diversity statistics show, the population is conserved across the Irish samples, but Warwick soil gives a completely different diversity profile. This is shown by both sets of phylogenetic trees where the majority of species from Warwick soil group independently from the Irish species and it is therefore possible that *Mycobacterium* species including *M. bovis* are localised to particular environments with a few exceptions. The data acquired in this research confirms that the presence of *M. bovis* in an environment is a direct result of exposure of soil to infected animals and that it does not have a widespread presence (as results given in **Chapter 5** will confirm).

One problem associated with any sampling regime and subsequent testing, whether that is using culture or molecular techniques to establish diversity, is that of bias. In this case there was a concern that the approach taken would skew the results given as it has been shown that DNA extraction and PCR is inherently biased towards some sequences and also biased towards detecting sequences that are present in greater numbers than others (Stach *et al.*, 2001; LaMontagne *et al.*, 2002). Frostegård *et al.* (1999) found that genus diversity in clone libraries of total community DNA obtained using several cell lysis and DNA extraction methods changed according to the method used. Anderson *et al.* (2003) showed that four different primer sets targeted to the 18S rRNA and internal transcribed spacer genes of fungi gave different diversity profiles when used in a PCR with the same total community DNA. Although theoretically PCR can detect 1 gene copy in a reaction, in reality single or low copy number genes can be swamped in the reaction by other more numerous genes (Lueders and Friedrich, 2003). An apparent bias was observed in this research when using PCR primer set JSY16SF/R. Although all mycobacteria tested proved to be PCR positive when the reaction was carried out on single species chromosomal DNA, when used on soil DNA the reaction was biased towards fast-growing mycobacteria. This could be due to the majority of fast-growing species having two copies of the 16S rRNA encoding gene in their chromosomes (Ninet *et al.*, 1996), or

possibly due to saprophytic fast growers being more suitably adapted to the soil environment and present in higher numbers (Govindaswami *et al.*, 1995). Using this primer set only Warwick soil and one A7 clone were represented in the slow-grower portion of the phylogenetic trees. Shared and diversity species statistics confirm this bias, with there being a relatively large difference between expected and observed diversity in the Irish soils compared to Warwick soil. To overcome this a second set of primers was developed to specifically target the slow-growing mycobacteria. In these phylogenetic trees (successfully devoid of any fast-growing species) species are present, which were not previously detected. In particular *M. bovis* related species were detected. The diversity statistics in this method show a much greater correlation between observed and estimated diversity than before, indicating that the primers are more successful in representing the diversity of their target. This is in important factor to consider when designing primers to detect such diverse genera, and suggests that in the case of the mycobacteria specific groups of primers would give a more accurate representation.

The diversity statistics used also show variation among the results given, with differing values of expected diversity present. In both primer sets, Bootstrap gives the closest value of expected diversity to observed diversity with ACE giving the largest difference. MMruns and MMmeans give much greater diversity estimators as they use a different basis on which to calculate the statistics, with the addition of a second sample (in this case A7) skews the curve. This is an inherent problem in the calculation as the addition of a highly diverse sample raises the curve significantly (Raaijmakers, 1987). This is due to these statistics randomising the pooled samples and averaging the randomisations rather than randomising the individual species in a sample as the others do. This results in diverse samples being hugely overestimated as the diversity is increased by a factor over each randomisation.

The RNA analysis of the *M. bovis* BCG microcosms (**Chapter 3**) indicated the presence of 16S rRNA sequences in over long periods of time, taking this in conjunction with the DNA data, *M. bovis* BCG can remain viable in soil microcosms for up to 15 months in certain conditions, although they can no longer be cultured. As no mRNA was detected in

these microcosms this could be due to the cells entering a dormanct (viable-nonculturable) state similar to *M. tuberculosis* in the latter stages of primary infection, where reduced oxygen tension was thought to trigger the tubercle bacillus to enter a state of dormancy (Cunningham and Spreadbury 1998). This also occurred in *M. bovis* BCG, again due to hypoxia-induced dormancy (Boon *et al.*, 2001, Boon and Dick, 2002). Alternatively RNA detection limits and cell culture detection limits are approximately three log fold higher than DNA detection limits, and therefore these cells could have been viable and active, but present at such low numbers that detection of mRNA was impossible.

Chapter 5: Survival and Distribution of *Mycobacterium bovis* on a Farm-land Site

5.1 Introduction

The transmission routes of bovine tuberculosis are the focus of much research (DEFRA 2003), in particular, transmission between cattle and potential wildlife reservoirs, such as the badger have been studied, although other routes are also thought to be involved. One transmission route is believed to be through cattle to cattle contact (analogous to horizontal transmission of human tuberculosis) (Phillips *et al.*, 2003). This route has been reported to be responsible for between 10 and 15% of all individual bovine tuberculosis cases in the UK. The presence of single reactors in herds means that this route is not common in disseminating tuberculosis, possibly due to the disease being detected early in the infection, so *M. bovis* cells would not reach the high numbers needed for transmission (the minimum infectious dose is believed to be over 10^6 cells/ml) (Chambers *et al.*, 2001). However, other studies report the contribution of cattle to cattle transmission to be much higher, although not the major route of infection (Goodchild and Clifton-Hadley, 2001). The second major route of tuberculosis to cattle is believed to be via exposure of cattle to infected Eurasian badgers (*Meles meles*). There has been no qualifying statement released as to the nature of this transmission, only that a possible link may be present (Krebs report, 1997) with reports varying between badgers transmitting tuberculosis to cattle, and cattle transmitting tuberculosis to badgers (Hutchings and Harris, 1997). There is also evidence that a secondary wildlife reservoir is present, indeed the reservoir is thought to be the brushtail possum (*Trichosurus vulpecula*) in New Zealand (Corner *et al.*, 2002) and the white-tailed deer (*Odocoileus virginianus*) in the US (Schmitt *et al.*, 2002). In both the UK and Ireland the general consensus is that contact with infected badgers is the predominant route of transmission (Olea-Popelka *et al.*, 2003). Badgers excrete large quantities of viable *M. bovis* cells into the environment through urination, defecation and feeding activities (Chambers *et al.*, 2002), so *M. bovis* is likely to be present in environments used by infected badgers. Although the effect of disinfectants on *M. bovis* in soil and other environmental samples i.e. slurry, silage, has previously been investigated (Scanlon and Quinn, 2000), there has been little analysis of naturally occurring *M. bovis* in farm sites. Most research has concentrated on artificial inoculation of *M. bovis* into environmental samples and monitoring survival in these. Duffield and Young (1985) inoculated *M. bovis* into dry and moist soils under a variety of

environmental conditions. The organism could be re-extracted using the decontamination method at four weeks after inoculation in 80% shade, but not from soils exposed to sunlight. This research on the survival of *M. bovis* in soils and slurry employed the use of culture techniques (the problems associated with this has been previously discussed in **Chapters 3** and **4.** In other research, Tanner and Michel (1999) exposed lung and lymph node tissue of African buffalo (*Syncerus caffer*), which was macroscopically infected with *M. bovis,* to the environment by placing the samples directly onto soil, or by burying at different depths. Survival of *M. bovis* was monitored over time, with results showing that culturability was lost after 6 weeks in all experiments. The deeper the tissue was buried the lower the culturability was. The conclusion was reached that this loss of culurability equated to death of the bacteria. Faecal samples were also spiked with *M. bovis* cells, with culturability remaining for 4 weeks. The artificial nature of this research is apparent, as the *M. bovis* cells would have been in granulomas in the lung tissue, and would not accurately represent their state had they been introduced to the soil through urination and defecation (the most probable route of contamination) (Chambers *et al.*, 2002). Interestingly, these results do correlate with the loss of culturability of *M. bovis* BCG as given in this thesis in **Chapter 3,** where loss of culturability occurred between 6 and 8 weeks.

There has been little research concerning soil as a possible reservoir for *M. bovis* or as a source of cattle infection, considering the extensive research on the survival of the related species *M. avium* in the environment. Studies have revealed that *M. avium* is commonly isolated from environmental samples e.g. soil, water and dust particles (Torkko *et al.*, 2002, Ichiyama *et al.*, 1988). This organism is an extremely important pathogen with studies showing that between 25 and 50 % of US and European AIDS patients with profound immunodeficiency are infected (Moore and Chaisson, 1996). Strains with the same serotype have been isolated from both patients and their environment, and it is believed that the environment provides a transmission route for the organism (Saito *et al.*, 1990). The majority of *Mycobacterium* spp. are commonly occurring soil saprophytes, and *M. bovis*, although now an intracellular pathogens, retains many genes dating back to a previous saprophytic existence (Brosch et al., 2002). Two non-bacterial species may

also be involved in the concentration and survival of *M. bovis* in the environment. *M. avium* has been shown to survive and replicate in *Acanthamoeba polyphaga* and remain viable in amoebal cysts. Once ingested by the amoeba (in this case *Acanthamoeba castellanii*) the bacteria became resistant to several antibiotics; rifabutin, azithromycin, and clarithromycin (Miltner and Bermudez, 2000), and also became more infectious, this is thought to be due to the triggering of the expression of survival genes (Cirillo *et al.*, 1997). As *M. bovis* is also an intracellular pathogen, infecting macrophages, it has been postulated that it could survive and replicate in amoeba in a similar fashion to *M. avium* (Cirillo *et al.*, 1997). Recent research has confirmed this with *M. bovis* surviving ingestion by *Acanthamoeba castellanii*, although strains of *M. bovis* BCG did not (Taylor *et al.*, 2003). Secondly, earthworms (*Oligochaeta* and *Lumbricidae*) concentrate *M. avium* cells in their intestinal tracts as they feed in the soil environment, as these are the badgers primary food source, it is hypothesised that this may be a transmission route of tuberculosis to badgers, if *M. bovis* is concentrated in the same manner (Fischer et al., 2003).

Molecular methods to monitor presence and survival of *M. bovis* in environmental samples has not been previously attempted, although they have been used to determine active infection, and presence of cells in clinical samples. PCR has been used both in human and bovine tuberculosis for providing a rapid method for determining presence of *M. bovis* (and *M. tuberculosis*) (Sarmiento *et al.*, 2003). PCR-based typing methods have also been developed for determining the epidemiology and etiology of infection, primarily developed for human tuberculosis these typing techniques have been used in bovine tuberculosis cases (Cousins *et al.*, 1998). One method involves spacer oligonucleotide typing (spoligotyping) and detects the presence or absence of spacers of the direct repeat locus of the *M. bovis* genome (Aranaz *et al.*, 1996), using PCR followed by probe hybridisation. Another typing method is fingerprinting based on variable numbers of tandem DNA repeats (VNTR)(Filliol *et al.*, 2000). This method is used to determine the numbers of direct DNA repeats in the genome using PCR and gives specific banding patterns for individual strains (Frothingham and Meeker-O'Connell 1998). The detection of presence and location of IS*6110* sequences in a strain can also be

carried out for typing strains (Fang *et al.*, 1998) This insertion sequence has been shown to be locationally stable in the chromosome over long periods of time and through the use of PCR followed by RFLP, banding patterns can be established for individual strains (Costello *et al.*, 1999). All of the typing methods described require a single culture of the strain being investigated and therefore preclude their use in total community DNA. As it would not be known if a single strain was present in a soil sample and the likelihood that the typing methods would detect genes from indigenous soil bacteria, specific banding patterns would not be generated. Typing methods can be used to detect infection in clinical samples due to the extremely low possibility of the presence of more than one infective strain. Without the use of cell extraction and culture from soil, there is no way of determining if one or multiple strains are present and therefore typing would not give an accurate result if used on community DNA. Molecular techniques can be used to give a presence/absence result of the target organisms (in this case *M. bovis*) and also can be used to determine the nature of the cells, and their viability using RNA analysis (Keer and Birch, 2003). These methods have been employed in this research to determine the presence or absence of *M. bovis* in soil samples.

5.2 Aims

To monitor the presence or absence of genes relating to *M. bovis* in soils from a farm with a recent herd breakdown of bovine tuberculosis. This would be carried out using total community DNA extraction, followed by PCR, from samples taken at multiple time-points to determine persistence of the genes.

To determine the source of any DNA sequences detected, i.e. if they were from dead cells, dormant cells, or active cells, using total RNA extraction from the soil samples followed by RT-PCR targeted to *mpb64*, *mpb70* and to *M. bovis* 16S rRNA sequences.

5.3 Sampling of a farm-site in Ireland

The farm chosen for monitoring survival of *M. bovis* in soil was in County Louth, Ireland. This farm had suffered from two separate cases of bovine tuberculosis infection, one year apart, with the history of the infection on this farm fully documented. This work

was carried out in collaboration with Doctor Eamonn Gormley (University College Dublin, Ireland).

5.3.1 History of infection on the farm

The farm is located at OSI (Ordinance Survey Ireland) reference: O1089 (Fig 5.1), and the time-line of events i.e. when infection occurred, removal of animals, and so on, is known. This time line is given below.

Pre-January 2000

The complete cattle herd was placed under movement restriction after reactors were found.

Early January 2000

Reactors were confirmed as TB cases, 30% of animals were skin test reactors, further testing of the remaining herd resulted in the entire herd being removed for slaughter as a precautionary measure.

Late January 2000

A pro-active cull was carried out on badgers in sets in contact with the farm. The majority of animals were removed immediately. Monitoring for badger activity continued from this period with all animals removed from the farm by the close of January 2000.

February 2000

Post-mortem examination were carried out on badger carcasses revealing more than 20% had tuberculous lesions. Further culture and typing experiments revealed the infection was caused by the same strain of *M. bovis* as the cattle infection.

April 2000

Soil samples were taken from the farm and transported to Warwick University for examination.

August 2000

The farm was restocked with cattle the animals supplied from farms with no apparent presence or risk of bovine tuberculosis.

January 2001

During routine testing several of the new animals were declared as unconfirmed reactors. Further examination of the slaughtered cattle, confirmed their reactor status. Infected animals were culled, with remaining cattle placed on movement restriction. No badger activity was monitored at this point.

April 2001

Movement restrictions were placed on farms, due to the Foot and Mouth outbreak in Northern Ireland, which meant that no sampling (or even access to the farm) was possible for the next 8 months.

November 2002

The complications of the Foot and Mouth outbreak, and removal of access to the farm by the owner, meant continuing sampling was delayed until this point. In early November a second set of soil samples was taken and transported to Warwick University.

February 2003

The first sighting of new badger activity on the farm was noted, these animals were trapped and removed. The herd was tested and declared TB negative. Cattle restrictions were lifted and the farm declared as TB free.

The exact timing of these events is extremely important in determining the relationship between presence of *M. bovis* in the environs of the farm and the possible points of contamination. The timing of these events gives a period of 4 months with no animal activity (cattle and badgers) prior to the first sampling and 23 months, with no badger activity and no infected cattle activity for the second (although non-infected cattle were present).

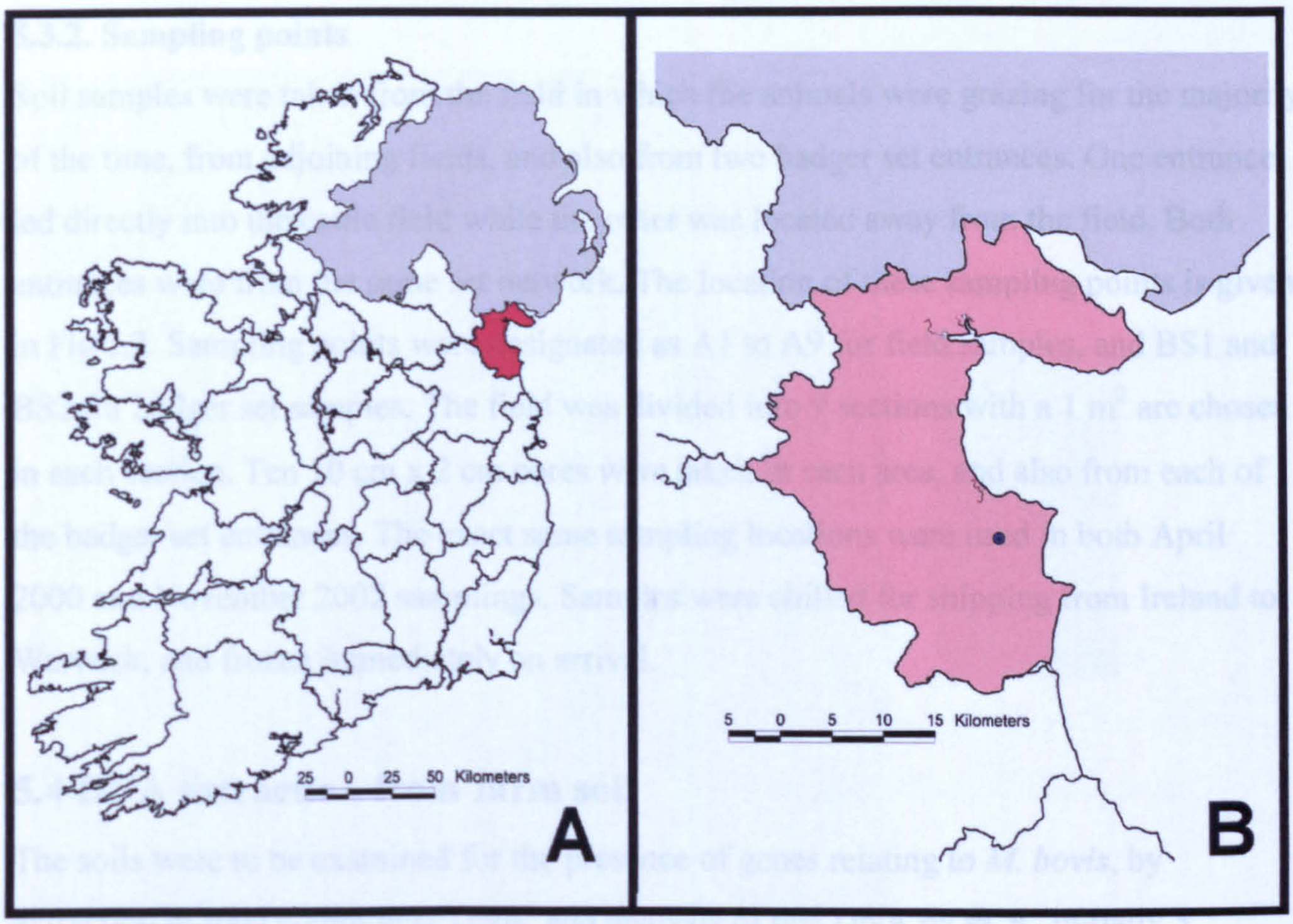


Fig 5.1 Location of sampling site. Maps give location of A) County Louth and B) the area of sampling within the county. OSI (Ordinance Survey Ireland) reference: O1089

5.3.2. Sampling points

Soil samples were taken from the field in which the animals were grazing for the majority of the time, from adjoining fields, and also from two badger set entrances. One entrance led directly into the cattle field while the other was located away from the field. Both entrances were from the same set network. The location of these sampling points is given in Fig 5.2. Sampling points were designated as A1 to A9 for field samples, and BS1 and BS2 for badger set samples. The field was divided into 9 sections with a 1 m^2 are chosen in each section. Ten 10 cm x 2 cm cores were taken in each area, and also from each of the badger set entrances. The exact same sampling locations were used in both April 2000 and November 2002 samplings. Samples were chilled for shipping from Ireland to Warwick, and frozen immediately on arrival.

5.4 DNA extraction from farm soil

The soils were to be examined for the presence of genes relating to *M. bovis*, by extraction of total community DNA, and analysis of this DNA by PCR. Initially 3 methods of DNA extraction were employed. Each method involved a lysis step (bead beating) followed by incubation with lysozyme, SDS and proteinase K, then precipitation with potassium acetate and PEG 6000. Each method then had a different DNA clean-up step: Chelex, Sephadex or Sepharose 4B (as in **2.6.2**). Problems were encountered with the use of both Chelex and Sephadex methods as they gave either no DNA, or very impure DNA (Fig 5.3). Both DNA samples prepared by these methods did not give PCR products even when spiked with *M. bovis* BCG DNA. This meant that some PCR inhibitors were being carried over from the extraction, possibly in the form of humic acids, as the DNA samples appeared to a have a visible colour tinge, even after several passes through Chelex and Sephadex.

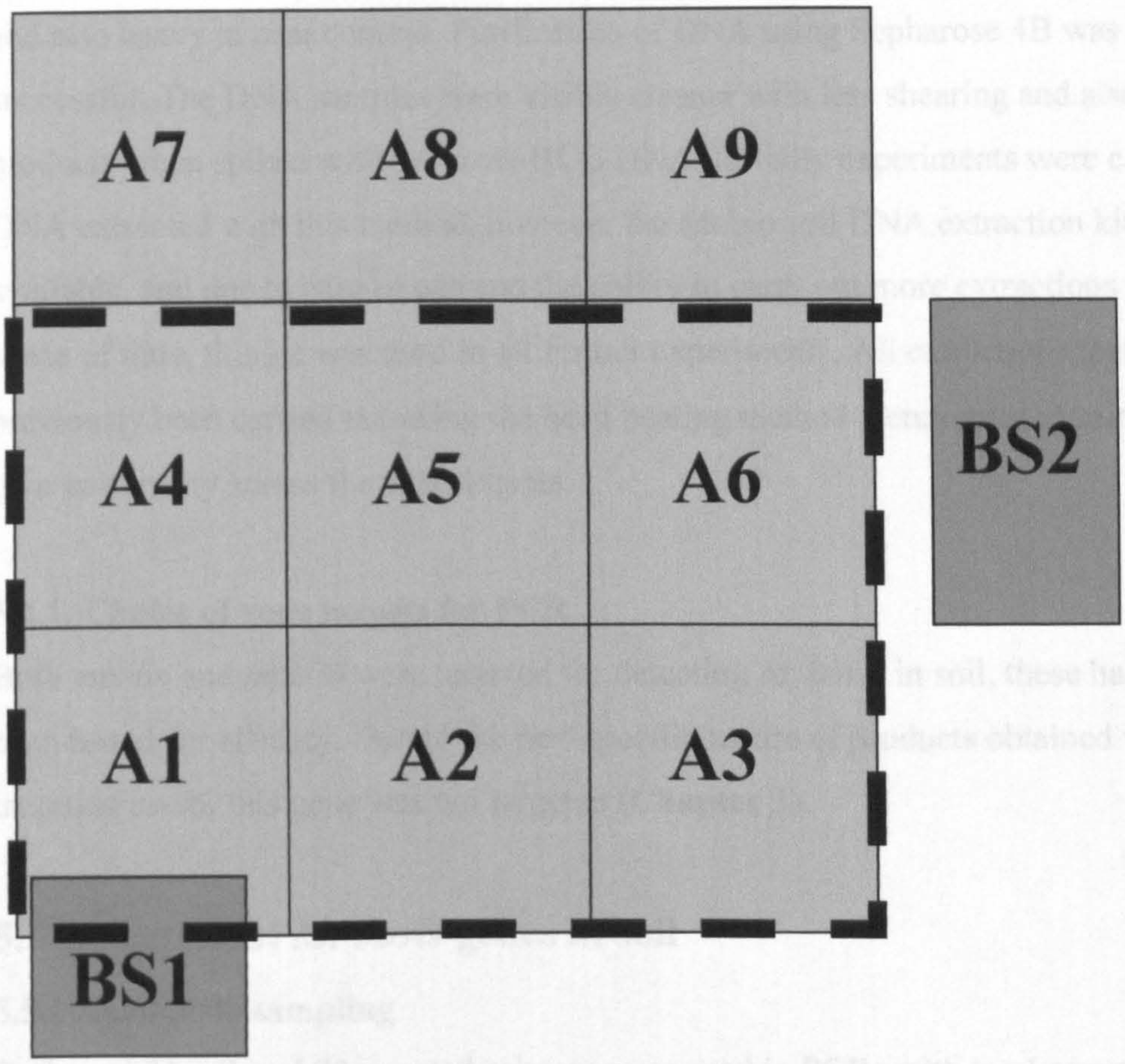


Fig. 5.2. Designation and location of soil sampling points. Dashed line indicates the boundaries of the field where the cattle were housed. Approx. area of field = 1 acre.

This contamination was perhaps due to the soils having a high organic content, being wet and also heavy in peat content. Purification of DNA using Sepharose 4B was more successful. The DNA samples were visibly cleaner with less shearing and also gave PCR products when spiked with *M. bovis* BCG DNA. Initially experiments were carried out on DNA extracted with this method, however the Mobio soil DNA extraction kit became available, and due to ease of use and the ability to carry out more extractions in a shorter space of time, this kit was used in all further experiments. All extractions that had previously been carried out using the bead beating method were repeated using this kit, to give uniformity across the experiments.

5.4.1. Choice of gene targets for PCR

Both *mpb64* and *mpb70* were targeted for detecting *M. bovis* in soil, these had previously been tested for efficacy. Due to the non-specific nature of products obtained when targeting *esat6*, this gene was not targeted (**Chapter 3**).

5.5 Detection of *M. bovis* genes in soil

5.5.1 April 2000 sampling

Both *mpb64* and *mpb70* targeted primers were used in PCRs with total community DNA extracted from soil. Specificity of the primers had already been determined, as had the presence/levels of the two antigen genes in a number of soils (Chapter 3). DNA was extracted from 1 g of each of the 110 soil cores for the April 2000 sampling using the Mobio extraction kit. PCR was then carried out on the DNAs using both *mpb64* and *mpb70* targeted primers. A representative result from this experiment is given in Fig 5.4. Analysis of the results from each of the PCRs revealed that no gene targets were present in soil from sampling points A3 to A9, in any of the cores. Target was detected for both primer sets in 90% of BS1 cores, 90% of BS2 cores, 90 % of A1 cores, and 80% of A2 cores (Fig 5.5).

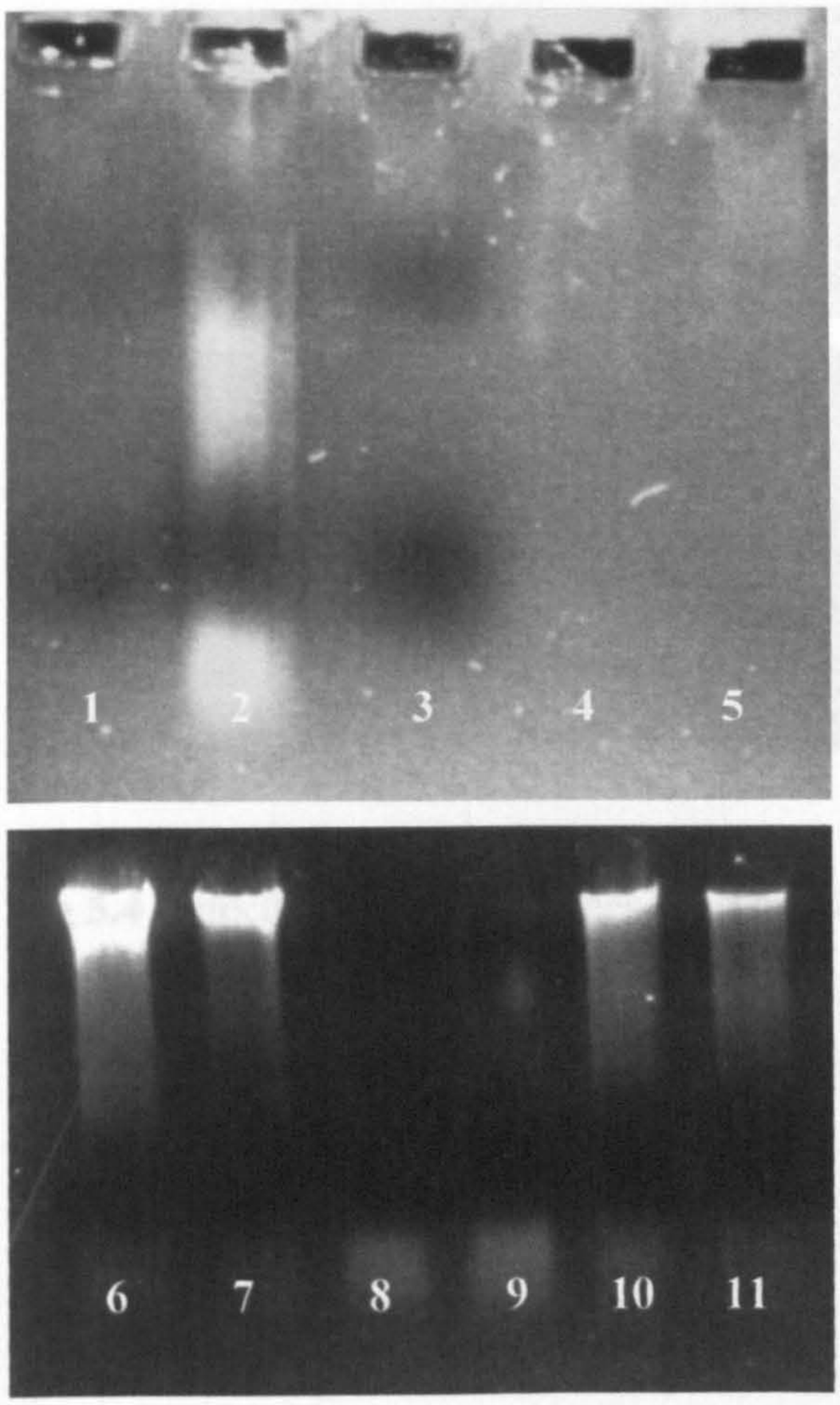


Fig. 5.3. DNA extractions from Irish soil sample BS1 using Chelex, Sephadex and Sepharose 4B purification methods. Lane order is 1,2,3,4,5) Chelex extraction, 6,8,9) Sephadex extraction, 7, 10, 11) Sepharose 4B extraction.

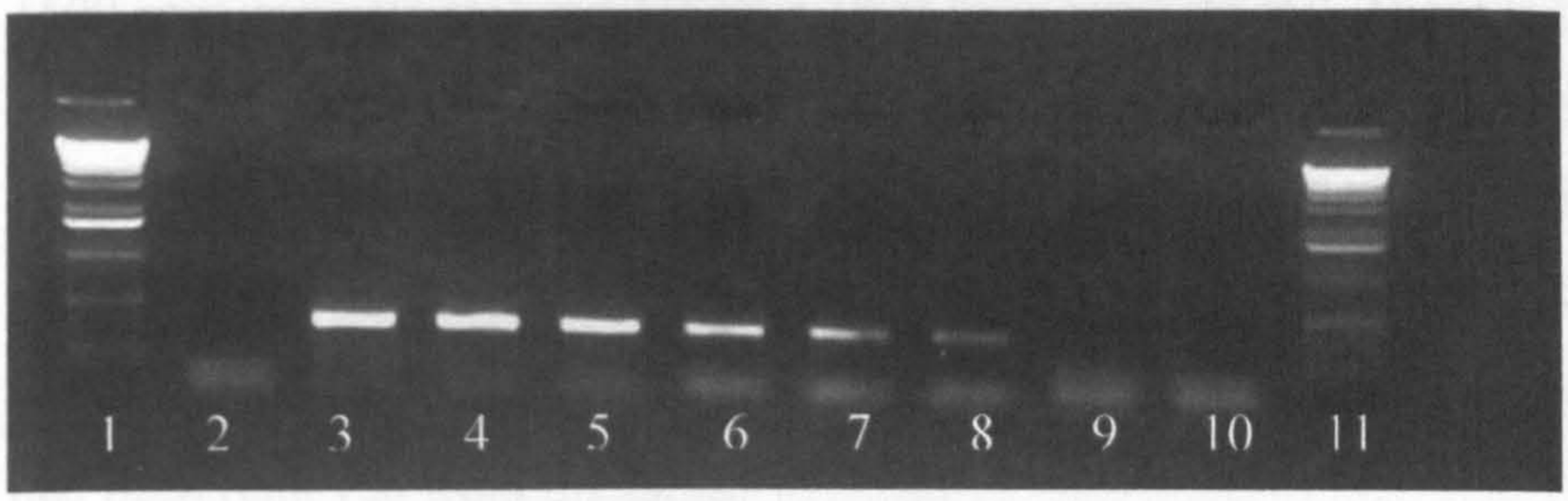


Fig 5.4 Detection of antigen genes in Irish soil samples targeting **A)** *mpb64***1, 11)** Molecular Markers. **2)** Negative Control.**3)** A6. **4)** A5. **5)** A4. **6)** A3 **7)** A2. **8)** A1. **9)** BS1. **10)** *M. bovis* BCG and **B)** *mpb70* (April 2000 sampling) **1, 11)** Molecular Markers. **2)** Negative Control.**3, 4, 5). 6)** BS. **7)** A1**. 8)** A2. **9)** A3. **10)** A4.

Quantitation was carried on the 35 positive PCR products as in **Chapter 3** again using the *M. bovis* BCG standards on each gel analysed. Individual quantitations for each sampling point were averaged and are given in Fig 5.6. BS1 gave the highest level of gene copies with 3600 gene copies/g soil, BS2 had 2500 gene copies/g soil, A1 gave 1500 gene copies/g soil and A2 gave 1000 gene copies/g soil. These values are given in gene copies rather than cells, as although every *M. bovis* strain studied so far has only one copy of the gene per cell, it cannot be stated for certain in this case, until the strain has been extracted from the soil and chromosomal DNA analysed. This experiment gives levels of gene copies present in soil 4 months after any potential contaminating source had been removed from the farm.

5.5.2. November 2003

Total community DNA was extracted from the 110 farm soil cores taken in November 2003. PCR was then carried out on the DNAs using *both mpb64* and *mpb70* targeted primers. Analysis of the results from each of the PCRs revealed that no gene targets were present in soil from sampling points A1 to A9, in any of the cores. Target was detected for both primer sets in 80% of BS1 cores and 80% of BS2 cores (Fig 5.5). Quantitation was carried on the 16 positive PCR products as in (Chapter 3) again using the *M. bovis* BCG standards on each gel analysed. Individual quantitations for each sampling point were averaged and are given in Fig 5.6. BS1 gave the highest level of gene copies with 2500 gene copies/g soil, with BS2 having 2100 gene copies/g soil. Again values are given in gene copies rather than cell numbers. This experiment gives levels of gene copies present in soil 21 months after any potential contaminating source had been removed from the farm.

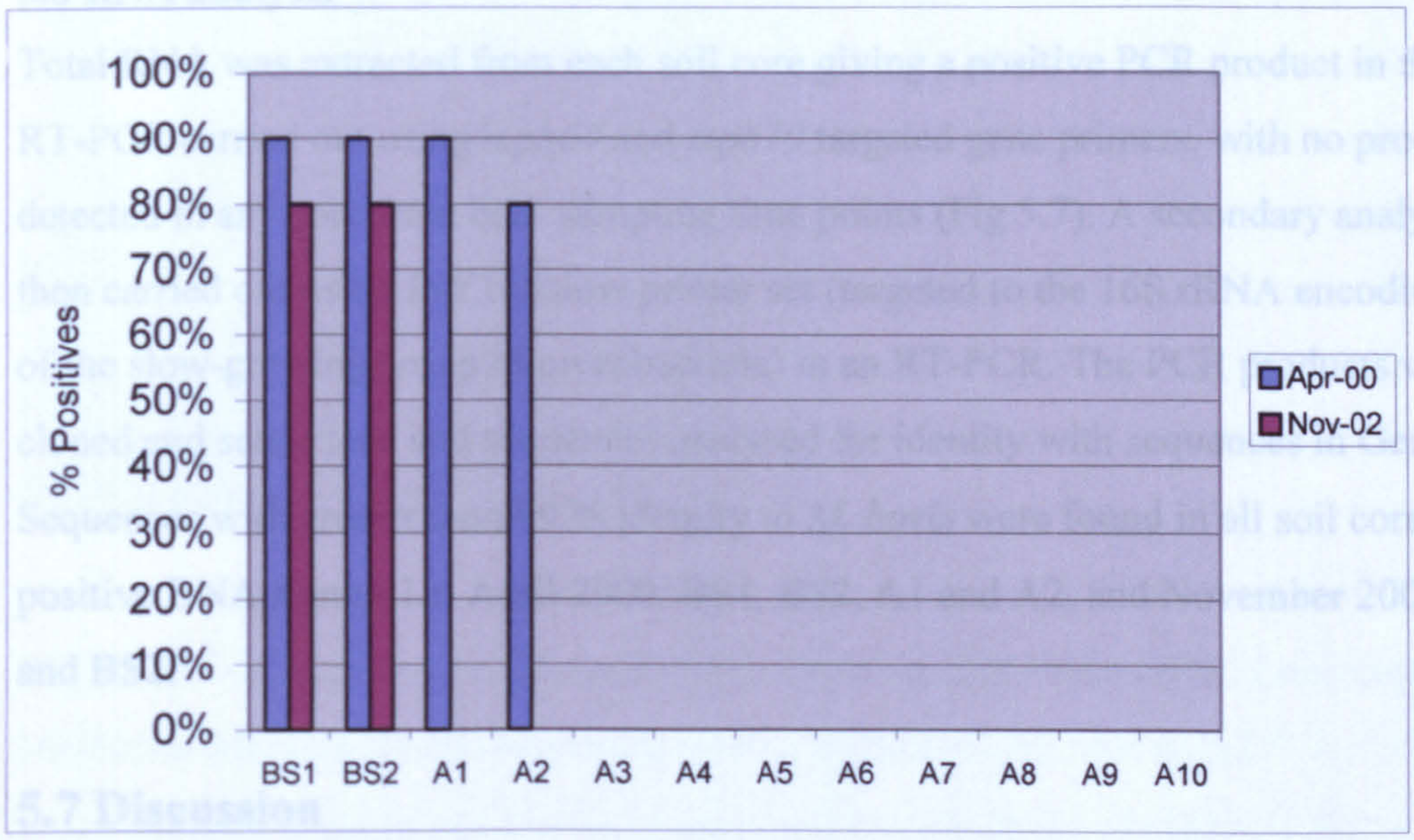


Fig 5.5 Number of individual Irish soil cores that gave PCR positive results for both *mpb64* and *mpb70*.

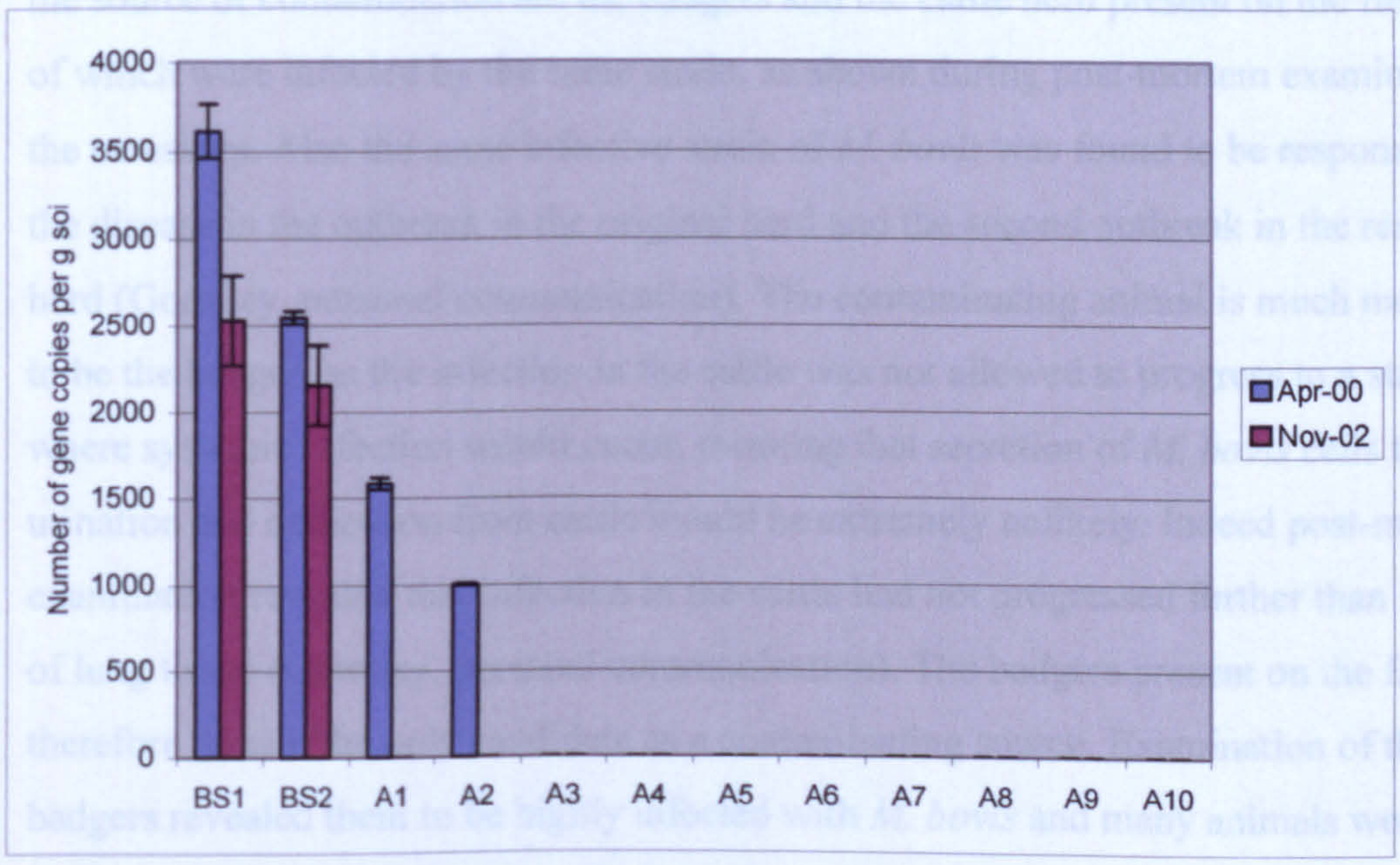


Fig 5.6. Levels of gene copies of both *mpb64* and *mpb70* in total community DNA extracted from Irish soil cores.

5.6 RNA analysis

Total RNA was extracted from each soil core giving a positive PCR product in **5.4** and RT-PCR carried out using *mpb64* and *mpb70* targeted gene primers, with no product detected in any core from both sampling time points (Fig 5.7). A secondary analysis was then carried out using JSY16Sslow primer set (targeted to the 16S rRNA encoding gene of the slow-growing group of mycobacteria) in an RT-PCR. The PCR products were then cloned and sequenced and sequences analysed for identity with sequences in Genbank. Sequences with greater than 99 % identity to *M. bovis* were found in all soil cores with positive DNA signals i.e. April 2000: BS1, BS2, A1 and A2, and November 2002: BS1 and BS2.

5.7 Discussion

The results given in this chapter prove the long-term survival of *M. bovis* DNA in soil following exposure to infected animals. The two animal species which could have been the source of contamination are the badgers and the cattle herd present on the fields, both of which were infected by the same strain, as shown during post-mortem examination of the carcasses. Also the same infective strain of *M. bovis* was found to be responsible for the disease in the outbreak in the original herd and the second outbreak in the restocked herd (Gormley, personal communication). The contaminating animal is much more likely to be the badger, as the infection in the cattle was not allowed to progress to a stage where systemic infection would occur, meaning that secretion of *M. bovis* cells through urination and defecation from cattle would be extremely unlikely. Indeed post-mortem examination revealed that infection in the cattle had not progressed further than infection of lung tissue (Gormley, personal communication). The badgers present on the farm therefore remain the only candidate as a contaminating source. Examination of the culled badgers revealed them to be highly infected with *M. bovis* and many animals were suffering with severe systemic infection. These animals would be excreting large numbers of *M. bovis* cells directly on to the soil.

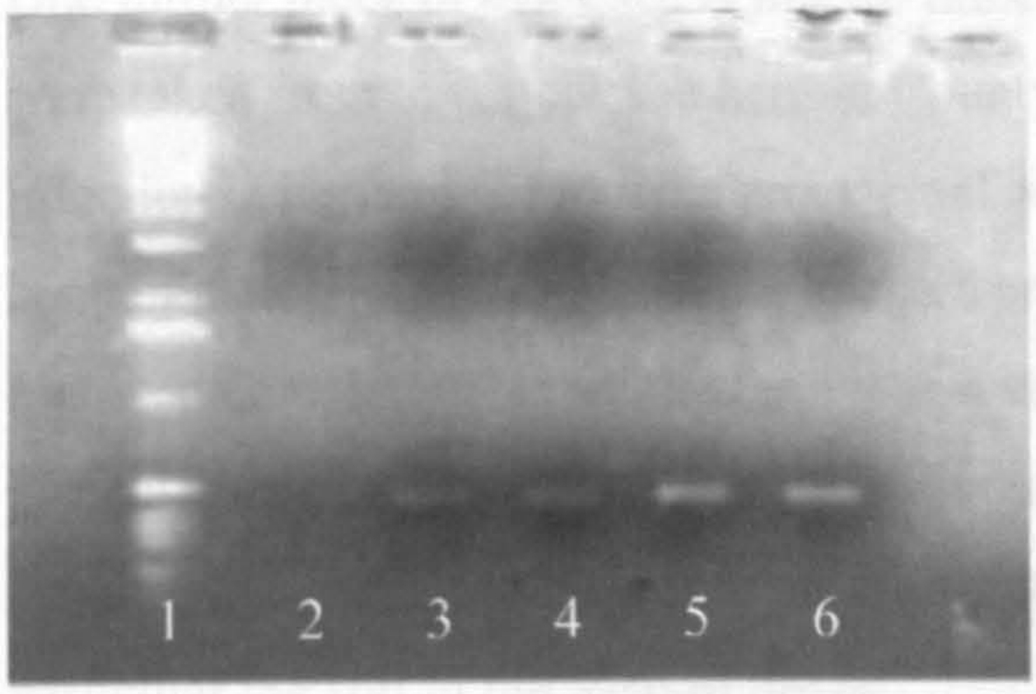


Fig 5.7. RT-PCR products obtained using primer set JSY16SslowF/R. Lane order is 1) Molecular Markers, 2) RT-Negative, 3) BS1, 4) BS2, 5) A1, and 6) A2.

One further piece of evidence is that no infection was noted in the herd from which cattle were taken from to restock the herd in County Louth. This rules out the possibility of the infection being brought in from an external source, meaning that the source of contamination must have been the badgers present on the farm. As these badgers were removed completely before the farm was restocked, the contamination must have been present outside of an animal host between outbreaks, and evidence presented in this research indicated that this reservoir of infection was the farm environment itself, in particular the soil in the pasture fields.

Analysis of DNA extracted from soil taken from the farm in April 2000 revealed that genes relating to M. *bovis* were found at four points, A1, A2, BS1 and BS2, with quantitation giving higher gene numbers in the two badger set samples. If the cattle were responsible for contaminating the soil it would be expected that positive signals would be found across the field i.e. in samples A3 to A6, however this was not the case. When badger set entrances have been found near to grazing cattle, it has been shown that the badgers rarely enter the main field area, preferring to feed in short grass areas found at the perimeter of fields. Direct badger-cattle interaction is very rarely observed indicating an intermediate route of infection between the two species. The soil environment would be the most obvious source of this intermediate route. Samples taken in November 2002 gave positive signals on only two sites, BS1 and BS2. Previously positive areas were now negative. As all infected badgers were removed this must have been a continuation of the original contamination as no obvious source of *M. bovis* cells was present. It could be argued that the restocked cattle herd could have reinfected the soil around the badger sets where the samples were taken, however although the herd would have had access to BS1, BS2 was completely isolated from the herd, and therefore could not have been re-contaminated by the cattle. Another alternative is the presence of a third animal reservoir. It has been shown that other mammalian species can carry *M. bovis* infection i.e. the fox (*Vulpes vulpes*), mink (*Mustela vison*), and the brown rat (*Rattus norvegicus*)(Delahay *et al.*, 2001; DEFRA, 2003). If a third animal host was present it would be extremely unlikely that the contamination would be limited to the badger sets, and that the presence of *M. bovis* cells would be detected in other areas.

There is also the possibility that the sampling and testing regime was not comprehensive, and that *M. bovis* genes were not detected in samples that were contaminated. No soil sampling regime and analysis using molecular methods can be totally comprehensive due to the variability of the soil environment. However, the percentage of samples, from one particular area, which were positive for *M. bovis* genes was between 80 and 90%, indicating the efficacy of the regime employed was high. Where negative results were obtained from a sample, DNA extraction and PCR was repeated throughout the sample. At no time was a previously negative sample found to be positive. Inhibition of PCR through humic contamination was also a possibility, which could have resulted in false negatives. With any negative sample PCR was carried out using primer set JSY16SF/R to test for PCR inhibition. All antigen gene negative samples were 16S rRNA gene positive. To test for primer specific inhibition all negative samples were spiked with *M. bovis* BCG DNA and PCR carried out. All the spiked samples were positive. With these results it is possible to conclude that all negatives found, were true negatives.

Results given in this chapter reveal the long-term survival of *M. bovis* genes in soil following initial contamination. It can be concluded that the badger is the only possible source of this contamination, and that no re-contamination occurred. Animals imported onto the farm were not infected prior to restocking and must have been infected once introduced. Evidence that the soil environment is a potential source of bovine tuberculosis is high, and further research should be carried out to determine the extent of the risk.

All of the evidence so far, has only revealed the presence of genes relating to *M. bovis*. Due to the fact that certain DNA sequences can remain intact in the soil environment for long periods of time it could be argued that the detection of these genes bears no relation to the infections of the animals on the farm. To determine the viability of the cells in the samples, RNA extraction was carried out followed by RT-PCR. The detection of 16S rRNA sequences in soil can be a good indicator of viable cells being present in a sample, as the turnover rate of RNA is much higher than DNA. The presence of mRNA in a sample is absolute confirmation of the viability of cells in a sample due to the extremely

low half-life of the molecule. In this research no mRNA was detected in any samples, which initially would indicate that the cells were dead. However, as described in **Chapter 3,** *M. bovis* cells have been shown to enter a dormancy state during times of biochemical and physical stress. Cells in this state would not be actively producing mRNA. Also PCR quantitation gave gene numbers no higher than 10^4 gene copies/g soil. This is on the limit of detection for mRNA in the soil samples, and so the extraction and detection methods would not be effective enough to detect mRNA species in the samples. Screening of samples positive for *M. bovis* gene sequences using RT-PCR revealed the presence of 16S rRNA sequences relating to *M. bovis* indicating that the cells are viable. This cannot automatically be deduced however, as research has shown that certain 16S rRNA species can survive for periods of time following cell death (Keer and Birch 2003). (Tolker-Nielsen *et al.*, (1997) presented evidence that 16S rRNA molecules were detected up to 1 week after terminal stressing of the cells (cold stress, acetic acid or ethanol treatment). The cells present in this farm soil samples would have been present for four months and it is unlikely for 16S rRNA sequences to have remained intact for this period of time, as they would have subject to degradation from extra-cellular nucleases present in the soil (Demaneche *et al.*, 2001).

Chapter 6: Final Discussion

6.1 Survival of *M. bovis* and *M. bovis* BCG

DNA from *M. bovis* and the vaccine strain *M. bovis* BCG (Pasteur) persisted for long periods of time in soil. Results presented in **Chapters 3 and 5** of this thesis show that presence of the organisms was detected up to 18 months after initial contamination of a natural soil environment, the first time such an observation has been noted. There have been numerous studies carried out on the isolation and characterization of *Mycobacterium* spp. from environmental samples, and *M. bovis* strains have never been successfully isolated. In research carried out by Donoghue *et al.* (1997), 750 putative mycobacteria were isolated from a farm in the South-West of England. After characterization of these isolates, none were found to group with *M. bovis*. In other research Little *et al.* (1982) monitored infected badgers, kept in an isolated yard, for tuberculosis infection and any consequent shedding of bacteria. As part of the experiment, environmental samples from the yard including badger faeces, soil, hay, scrapings from feeding bowls and water were examined for presence of *M. bovis*. Despite the animals concerned shedding high loads of *M. bovis* cells in urine and sputum, *M. bovis* was not isolated at any time. Duffield and Young (1985) could not detect *M. bovis* in artificially infected soil and faeces samples after four weeks. All three research projects were carried out using variations of the decontamination method (as in **Chapter 2**) supporting the evidence presented here, that this method severely reduces the mycobacterium population in a sample. The badgers examined by Little's group were shedding *M. bovis* intermittently at high levels, for between 165 and 1305 days before death from tuberculosis. It is unlikely that there would be no *M. bovis* present in environmental samples and points to there being problems involved in using a standard NaOH decontamination procedure for attempting to isolate *M. bovis* from soil. The decontamination method has however been successfully used on the isolation of non-tuberculous mycobacteria from soil and also the isolation of tuberculous species in clinical samples. Results given in **Chapter 3** indicate that the two fast-growing species tested, *M. smegmatis* and *M. phlei*, are less affected by the decontamination method than *M. bovis* BCG. This may show that fast growers in general are more resistant to the chemicals used, and certainly that BCG is compromised in its ability to withstand

chemical attack. Recent analysis has noted that BCG has massive genomic deletions compared to the parent strain *M. bovis*. It is also compromised in its ability to withstand the host immune response, hence its use as a successful vaccine (although it has the ability to replicate two or three times in a host, before removal occurs). It was therefore expected that BCG would not be able to withstand chemical toxicity as well as other species. This does not however, explain the lack of isolation of *M. bovis* in previous research (Little *et al.*, 1982; Duffield and Young, 1985). As the parent disease-causing strain, *M. bovis* is extremely effective at avoiding host immune response and attack from, for example, free radicals and reactive nitrogen factors in macrophages and leucocytes (Toossi 2002). It would be expected that *M. bovis* would show a higher resistance to the chemicals used in the decontamination procedure and it is surprising that naturally occurring *M. bovis* has never been isolated from the environment. One possible cause of this is that the cells may become compromised in some way, once they enter the soil environment. As stated in **Chapter 4**, the majorities of fast growers are soil organisms, and as such are more suited to this environment than the intracellular pathogen *M. bovis*. Perhaps the stresses on the cell present in the soil, e.g. desiccation stress, nutrient deprivation etc, lower the cells ability to resist the detrimental effect of the chemicals. It would be interesting to determine the effect that the decontamination and the individual chemicals have on *M. bovis* cells in soil, and this will be discussed further in **Section 6.3**.

A second reason for lack of *M. bovis* isolation from soil compared to isolation from clinical samples is that that of competition from other organisms. Clinical samples such as sputum, lung washes etc. would have a lower bacterial load compared to a soil sample. The contamination of growth media by these organisms would be less in a clinical sample, and it is more likely that *M. bovis* cells would be detected. Also the numbers of *M. bovis* cells in a clinical sample from a patient suffering detectable tuberculous disease would be much higher than in a soil sample (Wilsher *et al.*, 1999) (research presented here gave a *M. bovis* load in soil of between 10^2 and 10^3 cells/g soil). This larger number of target organisms would mean the successful isolation of *M. bovis* cells in a clinical sample would be much more likely than in a soil sample.

In **Chapter 3** it was concluded that *M. bovis* BCG DNA can persist in soil following inoculation of live cells into microcosms. Experiments carried out also show that this DNA is present in live viable cells, although they are not necessarily active. The presence of 16S rRNA sequences relating to BCG, and DNA turnover experiments, show that when culturability is lost, after approx. 60 days, live cells are present in the microcosms. This observation was also noted in results given in **Chapter 5**, with 16S rRNA sequences relating to *M. bovis* detected in farm soil samples, over one year after possible contamination. The lack of mRNA relating to the antigen genes *mbp64* and *mpb70* in these experiments firstly could indicate that the cells are in a dormant state, triggered by environmental stress (Hu *et al.*, 1999; O'Toole and Williams, 2003). However, it could be that mRNA was present, but at levels lower than the limit of detection, or that the target genes chosen were not expressed in the soil environment. As the exact function of the two genes *mpb64* and *mpb70* is not known (although they are believed to be involved in membrane lipid construction and transport (Harboe *et al.*, 1998)) it is difficult to determine if the genes would be expressed. To determine the state of the *M. bovis* and BCG cells in the soils it would be beneficial to try to detect constitutively expressed genes, specifically housekeeping genes, e.g. *recA* (Papavinasasundaram *et al.*, 2001). Alternatively the expression of genes involving in the dormant response such as the gene encoding for the α-crystallin protein (known to be expressed during the stress/dormancy response and suppresses the thermal denaturation of alcohol dehydrogenase) could be investigated (Yuan *et al.*, 1996). From evidence presented it can be concluded that both *M. bovis* and *M. bovis* BCG remain viable in soil for much longer than previously thought and may pose a risk to cattle, both in terms if infection in the case of *M. bovis*, and in terms of their reactor status in the case of BCG. This raises important questions as to the advice given to farmers following a case of bovine tuberculosis, and the procedures that should be carried out to remove any *M. bovis* cells present in the environs of the farm. Currently in the UK there is a requirement for disinfection of areas used by infected cattle including barns, sheds and water troughs, before new cattle are introduced (DEFRA 2003). There is, however, no requirement for disinfection of pasture and grazing land following herd infections. In Ireland again there is no requirement for disinfection of land, however there are recommendations that land should not be used for herds for 6

months after removal of infected cattle (The Department of Agriculture and Food, Ireland, 2003). The results presented here suggest that this should be readdressed and that the length of time should be increased.

Although the presence of viable *M. bovis* cells has been determined, there remains the question of how infectious these cells are and what danger these cells pose to cattle. It is conceivable that as these cells are viable and are present in relatively high numbers (10^2 to 10^3 cells/g), they have the ability to be infective. However, if they are in a dormant state, they could pose no risk. It therefore needs to be determined if these cells are infective. This would require an extraction and concentration step of the cells, and subsequent animal studies. The currently used model is a guinea pig aerosol model (Collins *et al.*, 2002), which is used partially due to the difficulties in using cattle in experiments (simply due to size) and also that results can be gathered much faster, due to the growth rates of the guinea pigs compared to cattle (Chambers *et al.*, 2001). The isolation would be difficult to carry out as no culture step could be employed. It is doubtful that any culture experiments would work, due to problems previously discussed, but if they were successful they would alter the nature of the *M. bovis* cells present, and would not accurately represent the environmental risk. There are methods for directly isolating cells from soil in particular using a Nycodenz density gradient (Courtois *et al.*, 2001; Berry *et al.*, 2003). This would extract the total cell mass however, and further steps would be needed to isolate *M. bovis* cells, possibly through the use of antibody-tagged cell sorting mechanisms (Surujballi *et al.*, 2002). The number of steps involved would have to be carefully monitored, as the processing again could alter the state of the cells present, not accurately representing the risk posed in the field. The use of immunomagnetic capture methods, would most likely give the best results. There are commercially, and non-commercially available immunomagnetic beads available which bind specifically to *M. avium* (Djonne *et al.*, 2003) and so it is possible to create specific antibody-bound beads for extraction of *M. bovis*. This method would require less processing steps and would more accurately represent the nature of the cells in the soil, once extracted.

6.2 16S rRNA analyis of soil communities

Analysis of the diversity of 16S rRNA sequences in four soil samples was determined (**Chapter 4**). Of these samples, three were taken from the same geographical area, a farm site in Ireland, and the remaining sample was from the Cryfield site at Warwick University (Wellington *et al.*, 1990). Sequences relating to the *M. bovis* group of the mycobacteria were detected in two samples, BS and A1, but not in A2 and W samples. The presence of these sequences in soil meant that four possible species were present in the sample; *M tuberculosis, M. microti, M. bovis or M. africanum.* Analysis of 16S rRNA sequences cannot successfully discriminate between these species due to the highly conserved nature of the encoding genes. The detection of the two antigen genes *mpb64* and *mpb70* in these samples, discounts the presence of *M. microti*, as *mpb70* is not present in this species (Cousins *et al.*, 1991). As the badgers on the farm were shown to be actively excreting *M. bovis* into the environment, and the lack of a source of contamination of *M. tuberculosis* and *M. africanum*, it is concluded that the sequences found related to *M. bovis.*

The three Irish soil samples had a high level of common species, with Warwick soil having a significant difference in diversity as determined though statistical analysis. Sequences relating to *M. avium* were detected in Warwick soil, but not in any other sample, similarly sequences relating to *M. conspicuum* and *M. interjectum*, The Irish samples contained sequences relating to *M. hiberniae*, which were not found in Warwick soil. These differences between sampling sites, and the shared community in the three Irish samples suggest that mycobacteria populations are conserved in local areas, and that there is little change in populations across samples taken from the same geographical locations. This natural diversity can be changed however due to external influences, in the case the introduction of *M. bovis* due to use of the land by infected animals. Statistical analysis of diversity and phylogenetic analysis of the sequence data, highlights the need for careful evaluation of results when using molecular tools in monitoring species diversity in soil. As previously described in **Chapter 4**, the primer set JSY16SF/R showed a marked bias towards 16S rRNA sequences from fast-growing mycobacteria.

This was confirmed in diversity analysis where expected diversity was much higher than observed diversity. The possible reasons for this bias have already been discussed, and suggest that when using DNA analysis for diversity measurement, a single primer set may not give an accurate representation of the diversity and a group of PCR primers should be used in conjunction. Due to the variable nature of PCR and of errors introduced in the reaction and in subsequent sequencing it is also important to use more than one target gene in analysis. In this case as well as using 16S rRNA analysis to determine the presence of *M. bovis,* the presence of two further genes *mpb64* and *mpb70* allowed the species to me more accurately defined.

6.3 Future work

The infective nature of the *M. bovis* cells detected in the Irish soil samples needs to be determined in order to quantify the risk that contaminated soils pose to cattle, and other animals. As described the cells would need to be isolated from the soils as quickly as possible and without the use of a culture step.

Further work could be carried out on the 16S rRNA clone libraries obtained from the Irish soil samples. As only a limited number of clones were analysed for the presence of sequences relating to *M. bovis*, it would be beneficial to continue sequencing these clones. As the sequences were obtained using RT-PCR, they would be a good indicator of activity in the soil. Using simple numerical analysis on sequence occurrence numbers in the libraries would give an estimate as to the predominantly active species in the soil. It would be interesting to compare these results to those obtained from clone libraries of the 16S rRNA gene, to determine if the species present in the largest numbers, are also the most active in soil.

Finally, at the time of writing a DEFRA-funded research project, in collaboration with Dr. Orin Courtney and Samantha Bryan (University of Warwick) has just been completed. This project is a continuation of work presented in this thesis and involves the use of the molecular techniques developed to determine the presence of *M. bovis* in multiple sites in the South-West of England. Over 1500 individual soil and badger faeces samples were taken from areas which had background data of conditions associated with

them, e.g. badger movements and numbers, cattle herd numbers, bovine tuberculosis status, and weather conditions. The samples originated from badger setts, badger latrines and cattle field sites and were anaylsed for the presence of *mpb64* and *mpb70* in extracted total community DNA. Positive signal were found in many of the samples, indicating a widespread presence of *M. bovis* in areas used by badgers. Initial analysis of results indicated that *M. bovis* was predominantly found in latrines and sett entrances that were close to the main sett network. Outlying entrances and latrines gave limited positive signals, as did field soils. Once fully analysed this research will give a better understanding of the presence and distribution of *M. bovis* in soil over a wide land area.

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Appendix A

Table A.1. Closest known sequence matches, and % identities for sequences obtained using primer set JSY16SF/R.

Sequence	Closest sequence match	% identity
GA11	*Mycobacterium* sp. IP20010531	95
GA112	*Mycobacterium moriokaense* strain CIP 105393	98
GA116	*Uncultured actinobacterium* clone SMS9.49WL	91
GA117	*Mycobacterium* sp. 1B(CD) clone *1*	98
GA118	Uncultured bacterium #0649-1118	98
GA119	*Mycobacterium sp.* HSC 1852	98
GA120	*Mycobacterium sp.* TA5	98
GA121	*Mycobacterium elephantis strain CIP 106831*	88
GA122	*M.hodleri*	98
GA124	*Mycobacterium sp. isolate JKD2379*	98
GA125	*Uncultured earthworm cast bacterium clone c211*	97
GA128	*Mycobacterium sp. isolate JKD2388*	93
GA129	*Uncultured earthworm cast bacterium clone c211*	98
GA13	*Mycobacterium sp. 1B(CD) clone 1*	96
GA131	*Mycobacterium sp. IP20010660*	97
GA132	*Mycobacterium moriokaense*	98
GA133	*Mycobacterium sp. JS623*	96
GA134	*Mycobacterium sp. TA5*	98
GA14	*Mycobacterium sphagni strain DSM 44076*	97
GA15	*Mycobacterium sp. TA5*	96
GA18	*Mycobacterium sp. TA5*	96
GA19	*Mycobacterium sp. TA5*	98
GA710	*Mycobacterium sp. JS624*	97
GA72	*Mycobacterium sp. TA5*	98
GA73	*Mycobacterium moriokaense*	98
GA74	*Mycobacterium sp. JS624*	98
GA75	*Uncultured earthworm cast bacterium clone c211*	95
GA76	*Mycobacterium moriokaense*	99
GA77	*Mycobacterium tokaiense*	98
GA78	*Mycobacterium sp. isolate JKD2379*	97
GBS1	*Mycobacterium hodleri strain CIP 104909*	92
GBS10	*Mycobacterium sp. isolate JKD2391*	96
GBS11	*Mycobacterium sp. NB01*	97
GBS12	*Mycobacterium sp. isolate JKD2391*	93
GBS13	*Mycobacterium moriokaense*	97
GBS14	*Mycobacterium sp. 1B(CD) clone 1*	99
GBS15	*Uncultured earthworm cast bacterium clone c211*	98
GBS16	*Uncultured earthworm cast bacterium clone c211*	93
GBS17	*Uncultured earthworm cast bacterium clone c211*	95
GBS18	*Mycobacterium fallax strain CIP 81.39*	89

GBS19	*Uncultured earthworm cast bacterium clone c211*	94
GBS21	*Mycobacterium sp. IP20010531*	98
GBS21	*Mycobacterium sp. IP20010531*	86
GBS22	*Mycobacterium sp. TA5*	93
GBS23	*Mycobacterium sp. TA5*	93
GBS25	*Mycobacterium goodii*	95
GBS27	*Mycobacterium tokaiense*	97
GBS29	*Mycobacterium wolinskyi strain CIP 106348*	89
GBS3	*Uncultured earthworm cast bacterium clone c211*	96
GBS31	*Pseudonocardia zijingensis*	98
GBS32	*Mycobacterium sp. isolate JKD2390*	94
GBS33	*Mycobacterium sp. isolate JKD2390*	97
GBS34	*Mycobacterium farcinogenes*	99
GBS35	*Mycobacterium sp. NB01*	94
GBS39	*Soil bacterium S103M1*	88
GBS4	*Mycobacterium tokaiense*	95
GBS40	*Mycobacterium poriferae strain CIP 105394*	81
GBS41	*Mycobacterium sp. isolate JKD2379*	96
GBS42	*Mycobacterium sp. Myc399*	73
GBS44	*Mycobacterium tokaiense*	95
GBS46	*Actinobispora yunnanensis*	93
GBS48	*Mycobacterium sp. TA5*	97
GBS6	*Mycobacterium sp. JS623*	98
GBS7	*Mycobacterium sp. JS624*	96
GBS8	*Mycobacterium sp. isolate JKD2388*	97
GBS9	*Mycobacterium moriokaense strain CIP 105393*	98
GW10	*Mycobacterium avium*	98
GW11	*Mycobacterium avium Myc373*	86
GW12	*Mycobacterium conspicuum strain CIP 105165*	98
GW14	*Mycobacterium sp. strain MCRO 8*	97
GW16	*Mycobacterium avium subsp. paratuberculosis*	86
GW17	*Mycobacterium conspicuum strain CIP 105165*	98
GW19	*Mycobacterium sp. strain MCRO 8*	97
GW21	*Mycobacterium conspicuum strain CIP 105165*	98
GW23	*Mycobacterium sp. strain MCRO 8*	97
GW4	*Mycobacterium aichiense strain JS618*	92
GW5	*Mycobacterium aichiense strain JS618*	97

Table A.2. Closest known sequence matches, and % identities for sequences obtained using primer set JSY16SslowF/R.

Sequence	Nearest sequence	% identity
GA11	Mycobacterium sp. IP20010531	95
GA112	Mycobacterium moriokaense strain CIP 105393	98
GA116	Uncultured actinobacterium clone SMS9.49WL	91
GA117	Mycobacterium sp. 1B(CD) clone 1	98
GA118	Uncultured bacterium #0649-1I18	98
GA119	Mycobacterium sp. HSC 1852	98
GA120	Mycobacterium sp. TA5	98
GA121	Mycobacterium elephantis strain CIP 106831	88
GA122	M.hodleri	98
GA124	Mycobacterium sp. isolate JKD2379	98
GA125	Uncultured earthworm cast bacterium clone c211	97
GA128	Mycobacterium sp. isolate JKD2388	93
GA129	Uncultured earthworm cast bacterium clone c211	98
GA13	Mycobacterium sp. 1B(CD) clone 1	96
GA131	Mycobacterium sp. IP20010660	97
GA132	Mycobacterium moriokaense	98
GA133	Mycobacterium sp. JS623	96
GA134	Mycobacterium sp. TA5	98
GA14	Mycobacterium sphagni strain DSM 44076	97
GA15	Mycobacterium sp. TA5	96
GA18	Mycobacterium sp. TA5	96
GA19	Mycobacterium sp. TA5	98
GA710	Mycobacterium sp. JS624	97
GA72	Mycobacterium sp. TA5	98
GA73	Mycobacterium moriokaense	98
GA74	Mycobacterium sp. JS624	98
GA75	Uncultured earthworm cast bacterium clone c211	95
GA76	Mycobacterium moriokaense	99
GA77	Mycobacterium tokaiense	98
GA78	Mycobacterium sp. isolate JKD2379	97
GBS1	Mycobacterium hodleri strain CIP 104909	92
GBS10	Mycobacterium sp. isolate JKD2391	96
GBS11	Mycobacterium sp. NB01	97
GBS12	Mycobacterium sp. isolate JKD2391	93
GBS13	Mycobacterium moriokaense	97
GBS14	Mycobacterium sp. 1B(CD) clone 1	99
GBS15	Uncultured earthworm cast bacterium clone c211	98
GBS16	Uncultured earthworm cast bacterium clone c211	93
GBS17	Uncultured earthworm cast bacterium clone c211	95
GBS18	Mycobacterium fallax strain CIP 81.39	89
GBS19	Uncultured earthworm cast bacterium clone c211	94
GBS21	Mycobacterium sp. IP20010531	98
GBS21	Mycobacterium sp. IP20010531	86

GBS22	Mycobacterium sp. TA5	93
GBS23	Mycobacterium sp. TA5	93
GBS25	Mycobacterium goodii	95
GBS27	Mycobacterium tokaiense	97
GBS29	Mycobacterium wolinskyi strain CIP 106348	89
GBS3	Uncultured earthworm cast bacterium clone c211	96
GBS31	Pseudonocardia zijingensis	98
GBS32	Mycobacterium sp. isolate JKD2390	94
GBS33	Mycobacterium sp. isolate JKD2390	97
GBS34	Mycobacterium farcinogenes	99
GBS35	Mycobacterium sp. NB01	94
GBS39	Soil bacterium S103M1	88
GBS4	Mycobacterium tokaiense	95
GBS40	Mycobacterium poriferae strain CIP 105394	81
GBS41	Mycobacterium sp. isolate JKD2379	96
GBS42	Mycobacterium sp. Myc399	73
GBS44	Mycobacterium tokaiense	95
GBS46	Actinobispora yunnanensis	93
GBS48	Mycobacterium sp. TA5	97
GBS6	Mycobacterium sp. JS623	98
GBS7	Mycobacterium sp. JS624	96
GBS8	Mycobacterium sp. isolate JKD2388	97
GBS9	Mycobacterium moriokaense strain CIP 105393	98
GW10	Mycobacterium avium	98
GW11	Mycobacterium avium Myc373	86
GW12	Mycobacterium conspicuum strain CIP 105165	98
GW14	Mycobacterium sp. strain MCRO 8	97
GW16	Mycobacterium avium subsp. paratuberculosis	86
GW17	Mycobacterium conspicuum strain CIP 105165	98
GW19	Mycobacterium sp. strain MCRO 8	97
GW21	Mycobacterium conspicuum strain CIP 105165	98
GW23	Mycobacterium sp. strain MCRO 8	97
GW4	Mycobacterium aichiense strain JS618	92
GW5	Mycobacterium aichiense strain JS618	97